Characterization of Thi9, a Novel Thiamine (Vitamin B₁) Transporter from Schizosaccharomyces pombe

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Thiamine is an essential component of the human diet and thiamine diphosphate-dependent enzymes play an important role in carbohydrate metabolism in all living cells. Although the yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe can derive thiamine from biosynthesis, both are also able to take up thiamine from external sources, leading to the down-regulation of the enzymes involved in its formation. We have isolated the S. pombe thiamine transporter Thi9 by genetic complementation of mutants defective in thiamine biosynthesis and transport. Thi9 localizes to the S. pombe cell surface and works as a high-affinity proton/thiamine symporter. The uptake of thiamine was reduced in the presence of pyrithiamine, oxythiamine, amprolium, and the thiazole part of thiamine, indicating that these compounds are substrates of Thi9. In pyrithiamine-resistant mutants, a conserved glutamate residue close to the first of the 12 transmembrane domains is exchanged by a lysine and this causes aberrant localization of the protein. Thiamine uptake is significantly increased in thiamine-deficient medium and this is associated with an increase in thi9 mRNA and protein levels. Upon addition of thiamine, the thi9 mRNA becomes undetectable within minutes, whereas the Thi9 protein appears to be stable. The protein is distantly related to transporters for amino acids, γ-aminobutyric acid and polyamines, and not to any of the known thiamine transporters. We also found that the pyridoxine transporter Bsu1 has a marked contribution to the thiamine uptake activity of S. pombe cells.

Beriberi was the first disorder for which the term “deficiency disease” was used. It is caused by a lack of thiamine, a water-soluble vitamin (vitamin B₂), which was identified in feeding experiments with deficient birds more than 80 years ago. Thiamine plays a pivotal role as coenzyme in intermediary carbon metabolism. Its biologically active form, thiamine diphosphate (TDP), is the essential cofactor of transketolases and enzyme complexes involved in the oxidative decarboxylation of oxo-acids. Williams and Roehm (1) discovered that the crystalline anti-beriberi substance also promoted the growth of yeast, a hallmark finding that paved the way for the use of microorganisms in vitamin research.

Most bacteria, fungi, and plants are able to synthesize thiamine. The two structural moieties of thiamine, 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) and 5-(2-hydroxyethyl)-4-methylthiazole (HET), are assembled by separate pathways. These pathways are well established in bacteria but appear to be completely different in most eukaryotes (2, 3). HMP and HET are phosphorylated and condensed to thiamine monophosphate. In eukaryotes, thiamine monophosphate is first hydrolyzed to free thiamine before it is converted to its cofactor form, whereas in bacteria, thiamine monophosphate is directly phosphorylated to TDP. Some microorganisms, algae, protozoa as well as most higher eukaryotes require thiamine as a growth factor (4). In some cases, this requirement can be met by HET or HMP or a combination of both, but in other cases only supplementation of thiamine allows growth (4). The mammalian diet is rich in phosphorylated forms of thiamine, which are dephosphorylated before they are taken up (5).

In many organisms, the availability of thiamine is known to have profound effects on gene expression. In the yeast Saccharomyces cerevisiae, thiamine represses more than 60 genes, most of which are involved in thiamine metabolism (6–8). In the current model of thiamine sensing, TDP functions as the intracellular thiamine signal. TDP binds to Thi3, a protein with homology to pyruvate decarboxylases and this weakens the interaction of Thi3 with Thi2, a Cys2 zinc-finger motif containing protein. Together, this is thought to diminish the activity of the Thi2-Thi3 complex to act as a positive regulator of thiamine-controlled genes (9). The DNA-binding protein Pdc2, a transcription factor for the glycolytic enzyme pyruvate decarboxylase, appears to control the same set of genes and may link the production of TDP to the available carbon source (7). TDP also mediates gene regulation in bacteria. It binds with high affinity to a regulatory RNA structure encoded in the 5′-untranslated regions of thiamine biosynthetic operons, thus reducing the expression of the downstream structural genes by a riboswitch mechanism (10–12). In summary, most

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4 The abbreviations used are: TDP, thiamine diphosphate; HET, 5-(2-hydroxyethyl)-4-methylthiazole; HMP, 4-amino-5-hydroxymethyl-2-methylpyrimidine; EMM, Edinburgh minimal medium; ORF, open reading frame; HA, hemagglutinin; GFP, green fluorescent protein.
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microorganisms have evolved means to ensure that thiamine is not produced in higher amounts than required for TDP biosynthesis.

Thiamine-dependant organisms require efficient plasma membrane transport proteins for thiamine acquisition. In S. cerevisiae, thiamine uptake is a high affinity process (K_m = 0.18 μM) that accumulates the substrate and displays an acidic pH optimum (13). Whereas HMP appears to share the same uptake system with thiamine, HET uses a different permeation pathway (14). These results with intact S. cerevisiae cells likely reflect the activity of Thi7, the first thiamine transporter identified on a molecular basis (15, 16). Two paralogs of Thi7, Thi71 and Thi72, appear to have only a minor contribution to thiamine translocation (7, 15, 16). These three proteins belong to the FUR family whose other members catalyze the uptake of purines and pyrimidines (17) and are unrelated to thiamine transporters from bacteria or mammals (11, 18–22).

Thi4 appears to initiate and catalyze a complex series of reactions that use NAD+ as a carbon donor and result in the formation of adenosine dipospho-5-(β-ethyl)-4-methyl-thiazole-2-carboxylic acid (23, 24). Nmt1 is 62% identical to S. cerevisiae Thi5. Thi5 or its paralogs Thi11, Thi12, or Thi13 are necessary for the synthesis of the pyrimidine part that starts from pyridoxine phosphate (25). In addition to thiamine biosynthesis, sexual agglutination and zygote formation also start from pyridoxine phosphate (25). In addition to thiamine and one transformant produced by sequential gene disruptions using knock-out cassettes. For nmt2+, the gene was amplified by PCR and ligated with blunt ends into the SrfI site of pPCR-ScriptAmp (Stratagene). kanMX4 was excised from pFA6a-kanMX4 (37) with SmaI and EcoRI. The gene was amplified by PCR and ligated into a pUC19 derivative with a plasmidic region with homology to thi14. These mediated homologous recombination after transforming the PCR product into BY4742. The transformation mixture was plated on synthetic dextrose containing 120 μM thiamine and one transformant (CVY4, thi7Δ::kanMX4 thi71Δ::LEU2 thi72Δ::LYS2 thi4Δ::his5+) was used for complementation.

S. pombe strains used in this study were FY254 wild type (ade6-M210 can1-1 leu1-32 ura4-D18 h-1) (36), FY254 bsl1Δ::ura4+ (29), and ptr1–5 pho1–44 pho4-4? h-1 (27), which we will refer to as ptr1. Further derivatives of FY254 were generated by sequential gene disruptions using knock-out cassettes. For nmt2+, the gene was amplified by PCR and ligated with blunt ends into the SrfI site of pPCR-ScriptAmp (Stratagene). kanMX4 was excised from pFA6a-kanMX4 (37) with Smal and EcoRI and inserted into Scal and MfeI sites of nmt2+. To generate a knock-out construct for thi9+, the ORF was amplified by PCR and cloned into a pUC19 derivative with a single NotI site. An internal HincII/EcoRI fragment of thi9+ was genomically fused to kanMX4, which was excised from pFA6a-kanMX4 with Smal and EcoRI. The nmt2Δ::kanMX4, nmt2Δ::LEU2, and thi9Δ::kanMX4 deletion cassettes were inserted into NotI before transformation, which was performed as described previously (38). The majority of the strains used in this work resulted from sequential transformations of FY254 or FY254 bsl1Δ::ura4+ with the knock-out cassettes and were verified by PCR. The ptr1 nmt2Δ double mutant was generated by crossing ptr1 to FY254 nmt2Δ::kanMX4 followed by tetrad dissection and marker analysis. The genotype of the spore used for complementation was ptr1–5 nmt2Δ::kanMX4 pho1+ ade6-M210 ura4-D18 h-1 thi9+ was genomically fused to gfp or a triple HA tag using an integrative tagging strategy (39). In pBS-bsu1+::GFP and pBS-bsu1+::3HA (29), the bsu1+ coding sequence was replaced with a PCR fragment containing the thi9+ coding sequence without stop codon. In the resulting plasmids, thi9+ is followed by the tag sequence, the S. cerevisiae ADH1 terminator, and the LEU2 gene as transformation marker. The vectors were linearized with NsiI, which cuts 969

EXPERIMENTAL PROCEDURES

Media and Culture Conditions—Synthetic dextrose medium for S. cerevisiae contained 2% glucose and 0.67% yeast nitrogen base without amino acids (Difco). Thiamine-free synthetic dextrose was made of yeast nitrogen base without amino acids and without vitamins (BIO101) and was supplemented with all vitamins except thiamine in standard concentrations. Bacto-agar (Difco) was used for solidification. Liquid cultures of S. pombe were grown in Edinburgh minimal medium (EMM), a chemically defined medium for fission yeast devoid of thiamine (30). S. pombe strains were crossed on ME plates (3% malt extract, 2% agar). Growth assays were performed on synthetic dextrose plates with cell suspensions of A600 = 0.06, 0.006, and 0.0006. These were prepared in 96-well plates and transferred to agar plates using a steel replication device. Growth was recorded after incubation for 3 to 5 days at 30 °C. Nucleobases and amino acids were added as required.

Yeast Strains and Knock-out Mutants—S. cerevisiae BY4742 (Mato his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) (31) was used as wild type strain and was transformed by standard methods (32). To delete THI71, the open reading frame was amplified by PCR with specific primers and cloned into pLitmus28 (New England Biolabs). The LEU2 gene was first ligated as a XhoI-Sall fragment from YEp13 (33) into the Sall site of pUC19 (pUC19-LEU2 I) and from there inserted into the PstI and NdeI sites within the THI71 ORF. To delete THI72, a 340-bp long 5′- and 490-bp long 3′-region of THI72 was amplified from genomic DNA. LYS2 was excised from YDP-K (34) and inserted together with both PCR fragments into pUC19. The knock-out cassettes were released from the plasmids and subsequently transformed into BY4742 or BY4742 thi7Δ::kanMX4 (EUROSCARF) resulting in the thi71Δ::LEU2 thi72Δ::LYS2 double knock-out (CVY2) and thi7Δ::kanMX4 thi71Δ::LEU2 thi72Δ::LYS2 triple knock-out strain (CVY3). To delete THI4, the HIS3MX6 marker gene containing S. pombe his5+ was amplified from pFA6a-HIS3MX6 (35) with specific primers containing terminal regions with homology to THI4. These mediated homologous recombination after transforming the PCR product into CVY3. The transformation mixture was plated on synthetic dextrose containing 120 μM thiamine and one transformant (CVY4, thi7Δ::kanMX4 thi71Δ::LEU2 thi72Δ::LYS2 thi4Δ::his5+) was used for complementation.

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bp after the start ATG of thi9\(^+\), and transformed into \textit{S. pombe} wild type cells and \textit{ptr1} mutants. After homologous recombination, this results in tagged versions of thi9\(^-\), which are expressed from their own promoters. As a result of plasmid integration, the cells contain a second copy of thi9\(^-\), which lacks a promoter and will not be expressed. In \textit{ptr1} cells, tagging of thi9\(^-\) will result in a fusion protein that carries the E81K amino acid exchange.

\textbf{Plasmids—}Two \textit{S. pombe} gene libraries were used in this study. The cDNA library used for complementation of CVY4 was generated in the \textit{S. cerevisiae} multicopy vector pFL61, in which the inserts are sandwiched between the PGK1 promoter and terminator (40). The \textit{S. pombe} genomic library in plasmid pUR19 was published before (41). THI7 from \textit{S. cerevisiae} was overexpressed as GFP fusion protein. To this end, THI7 including 640 bp of the promoter sequence was amplified by PCR and inserted into the multicopy plasmid YEplac195-GFP. YEplac195-GFP is similar to YCplac33-GFP but contains a 2-fluorophenylhydrazine/thi9\(^-\) promoter and terminator (40). The THI72 fusion of thi9\(^-\) was generated in the study. The cDNA library used for complementation of CVY4 was published before (41).

\textit{S. pombe} genomic DNA that included only the coding sequence and was inserted into NotI sites of pFL61. Expression of bsu1\(^+\) in \textit{S. cerevisiae} was performed using p426-GPD (43).

\textbf{Uptake Experiments—}Uptake experiments in \textit{S. cerevisiae} strains were performed as described previously (44). Briefly, the standard mixture contained 500 \(\mu\)l of cells \((A_{600} = 0.5)\) in citric acid/phosphate buffer, pH 4.5, and was stirred at 30 \(\degree\)C. After pre-warming for 2 min, the cells were energized with 1% glucose and the experiment was started 1 min later by addition of the labeled substrate for a final concentration of 2 \(\mu\)M. The substrate was a mixture of \([^{3}H]\)thiamine (ART-0710; American Radiolabeled Chemicals, Inc.) and unlabeled thiamine with a specific activity of 0.19 Ci/\(\mu\)mol.

Transport assays with \textit{S. pombe} were performed with the bsu1\(\Delta\) mutant (29). The cells were inoculated into fresh EMM to \(A_{600} = 0.3\) and grown at 30 \(\degree\)C until \(A_{600} = 1\). After harvesting and washing with ice-cold water and EMM, the cells were suspended in EMM to \(A_{600} = 10\) and kept on ice. 500 \(\mu\)l of cells (diluted to \(A_{600} = 2\)) were used for the assay that was performed as with \textit{S. cerevisiae} cells. For uptake experiments in the absence of glucose, the cells were re-suspended in glucose-free EMM. Competitors were added to a final concentration of 20 \(\mu\)M together with the labeled substrate. HET was obtained from Fluka, pyrithiamine and all other competitors used in Fig. 4 were from Sigma and HMP was a generous gift from T. P. Begley. The uncouplers carbonyl cyanide \(m\)-chlorophenylhydrazone and carbonyl cyanide \(p\)-(tri-fluoromethoxy)phenylhydrazone were from Sigma and were added 3 min prior to adding the substrate.

\textbf{RNA Preparation and Northern Blotting—}FY254 wild type cells were grown in EMM without thiamine and nmt2\(\Delta\) mutants in EMM supplemented with 8 \(\mu\)M thiamine for 3 days at 30 \(\degree\)C with daily passages to deplete intracellular thiamine pools. Fresh EMM was inoculated with overnight cultures to \(A_{600} = 0.2\) and the cells were grown for 2 h before repression was started by adding thiamine to a final concentration of 1.2 \(\mu\)M. At each time point, 25 \(A_{600}\) units of cells were harvested and RNA was prepared using the hot phenol method (45). 10 \(\mu\)g of RNA were separated on formaldehyde gels and transferred to nitrocellulose membranes. The genes thi9\(^-\) and act1\(^+\) (actin) were used as probes and labeled with [\(\alpha\)-\(\beta\)]DCTP. After hybridization, the Northern blots were quantified with a phosphorimager and the actin signal intensities were used for normalization.

\textbf{Western Blotting—}For Western blots, 10 \(A_{600}\) units of cells were broken with glass beads in 100 \(\mu\)l of TE buffer (25 mM Tris/HCl, pH 7.5, 5 mM EDTA) containing protease inhibitors. The lysate was centrifuged (20,000 \(\times\) g, 20 min, at 4 \(\degree\)C) and the membrane pellet solubilized in 100 \(\mu\)l of SDS sample buffer at 42 \(\degree\)C. The samples were separated on two 12.5% polyacrylamide gels. One gel containing 10 \(\mu\)l of sample/lane was stained with Coomassie dye to serve as a loading control, the other containing 1 \(\mu\)l of sample/lane was transferred to a nitrocellulose membrane. The blot was incubated with a mouse monoclonal anti-HA antibody (Santa Cruz Biotechnology, sc-7392), followed by a peroxidase-coupled secondary antibody and SuperSignal chemiluminescence reagent (Pierce). Quantification of band intensities was performed with weakly exposed films with the MultiAnalyst software.

\textbf{RESULTS}

\textbf{Identification of the \textit{S. pombe} Thiamine Transporter—}The main transporter for thiamine in \textit{S. cerevisiae} is encoded by \textit{THI7} (YL237W, synonym \textit{THI10}), which shares high homology to two other ORFs, \textit{THI71} (YOR071C) and \textit{THI72} (YOR192C). We tested strains with deletions of these genes for their thiamine uptake activity. A \textit{thi71\Delta thi72\Delta} double mutant (CVY2) was similar to a wild type strain, indicating that the main transport activity derives from \textit{Thi7}. To generate the best possible starting situation for genetic complementation assays, we generated a \textit{thi7\Delta thi71\Delta thi72\Delta} triple mutant (CVY3). This strain was highly deficient in thiamine uptake (Fig. 1A), which is consistent with earlier findings that \textit{Thi71} and \textit{Thi72} have only a minor contribution to thiamine transport (7, 15, 16). To additionally block thiamine biosynthesis, \textit{THI4} was deleted in CVY3. \textit{Thi4} catalyzes an essential step in the HET biosynthetic pathway. Hence, \textit{thi4\Delta} mutants strongly depend on an external source of thiamine (46). The resulting quadruple mutant (CVY4) required at least 120 \(\mu\)M thiamine and showed no growth on plates with lower thiamine concentrations (Figs. 1B and 3B). When \textit{THI7} was reintroduced on a plasmid, this strain could again grow on 0.012 \(\mu\)M thiamine, confirming that \textit{THI7} alone is sufficient to support the growth of \textit{S. cerevisiae}.

To isolate the \textit{S. pombe} thiamine transporter, the \textit{S. cerevisiae} strain CVY4 was transformed with an \textit{S. pombe} cDNA expression library (40) and the cells were plated on media containing 0.12 \(\mu\)M thiamine. A total of 24 transformants were obtained and their library plasmids were isolated. When retransformed into CVY4, 16 of them complemented the growth phenotype. None of the plasmids allowed growth on thiamine-free plates, eliminating the possibility that they contained \textit{nmt2\(^+\)}, the \textit{S. pombe} ortholog of \textit{THI4}. A total of 10 complementing plasmids were sequenced and all of them contained the complete coding sequence of SPAC9.10. The remaining six plasmids produced
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The characterization and complementation of yeast mutants with defects in thiamine transport. A, S. cerevisiae wild type strain BY4742 (wt) and mutants lacking transporter genes THI71 and THI72 (CVY2) or genes THI7, THI4, and THI9 (CVY4). This strain contained either an empty plasmid (control), a YEpLac195-plasmid encoding a gfp-tagged version of THI7, or a pFL61 plasmid harboring the cDNA of S. pombe ORF SPAC9.10, which we refer to as thi9+.

B, growth assays of a strain lacking THI71, THI72, and THI9 (CVY4). This strain contained either an empty plasmid (control), a YEpLac195-plasmid encoding a gfp-tagged version of THI7, or a pFL61 plasmid harboring the cDNA of S. pombe ORF SPAC9.10, which we refer to as thi9+.

C, the terms “ptr1 mutants” or “ptr 1 gene” as used in this study do not refer to the gene encoding an mRNASPAC9.10, which we refer to as thi9+.

A second strategy to identify the S. pombe thiamine transporter made use of a ptr1 strain5 that was isolated as a mutant being resistant to pyrithiamine, a toxic structural analog of thiamine (27). Pyrithiamine resistance is caused by a lack of pyrithiamine uptake and has earlier been used in the cloning of the S. cerevisiae THI7 gene (15, 16). We first crossed ptr1 mutants with nmt2Δ mutants and analyzed the tetrads. In all complete tetrads, the resistance against pyrithiamine segregated 2:2, indicating that it was caused by a single mutation. One pyrithiamine-resistant spore that combined the ptr1 and nmt2Δ mutations was used in the experiments described below. Consistent with a role of ptr1+ in thiamine uptake, the ptr1 nmt2Δ double mutant was unable to grow on medium containing <12 µM thiamine, where both parental strains readily grew. As expected, the nmt2Δ single mutants grew on lower thiamine concentrations and ptr1 mutants showed full growth even when thiamine was absent (Fig. 1C). Importantly, generation of the ptr1 nmt2Δ double mutant facilitated a positive selection (growth on thiamine-limited media) instead of a negative selection (pyrithiamine sensitivity) strategy to screen for the S. pombe thiamine transporter.

We transformed the ptr1 nmt2Δ double mutant with an S. pombe genomic library (41). Most of the 67 transformants that appeared on plates containing 0.12 µM thiamine were also able to grow in the absence of thiamine, indicating that they contained plasmids harboring nmt2+ (Fig. 1D). For random samples, this was confirmed by PCR. Six of the transformants showed good growth with 0.12 µM thiamine but reduced growth at lower concentrations and this phenotype was confirmed for five plasmids when transformed back into ptr1 nmt2Δ mutants (Fig. 1D). Sequencing revealed that all inserts overlapped with each other and included the ORF SPAC9.10. Thus, two independent strategies to identify the S. pombe thiamine transporter have led to the same ORF, SPAC9.10, which we will refer to as thi9+.

5 The terms “ptr1 mutants” or “ptr 1 gene” as used in this study do not refer to the gene encoding an mRNASPAC9.10, which we refer to as thi9+. In B–D, growth was recorded 3 days after plating on media containing the indicated concentrations of thiamine.
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**thi9**<sup>+</sup> *Is Allelic with ptr1**<sup>+</sup>—To prove that thi9<sup>+</sup> and ptr1<sup>+</sup> are allelic, we generated an *S. pombe* thi9Δ knock-out strain. Similar to the ptr1 mutant, the thi9Δ strain was resistant to pyrithiamine (Fig. 2C). Wild type cells were sensitive to pyrithiamine when plated on thiamine-free media, but they were resistant when the plates also contained thiamine (Fig. 2C). This is likely caused by the repression of thi9<sup>+</sup> in the presence of thiamine (see below). Besides being resistant to pyrithiamine, the thi9Δ and ptr1 mutants did not display obvious additional phenotypes. When we crossed the ptr1 mutant with the thi9Δ strain and analyzed the progeny for pyrithiamine resistance, all 56 spores isolated from 14 complete tetrads were resistant to pyrithiamine, demonstrating that thi9<sup>+</sup> and ptr1<sup>+</sup> are allelic (data not shown). To confirm this result, we sequenced thi9<sup>+</sup> from the ptr1 mutant and found that the gene contained a point mutation that changed glutamate 81 to lysine (E81K; Fig. 2A).

**Localization of Thi9**—The intron-less thi9<sup>+</sup> gene codes for a protein with 591 amino acids. Highly homologous proteins can be found in *Schizosaccharomyces japonicus* and many fungal species including important human (*Aspergillus fumigatus* and *Cryptococcus neoformans*) and plant (*Botryotinia fuckeliana* and *Magnaporthe grisea*) pathogens (Fig. 2A). The *S. cerevisiae* transporters for γ-aminobutyric acid (Uga4), choline (Hnm1), polyamines (Tpo5), and histidine (Hip1) are the only functionally characterized orthologs of Thi9. These proteins are members of the ACT (amino acid/choline transporter) family within the APC (amino acid/polyamine/organofluorocarbon) superfamly (TC2.A.3.4) (47) of which Thi9 is a distant member. Although Thi9 shares only 23.1% identical amino acids with Uga4 and even less with the other transporters, proteins with high similarity to Thi9 were variably annotated as permeases for choline, polyamines, or basic amino acids. Besides its positive charge, however, thi9maes has only little similarity to these compounds. Neither is Thi9 from *S. pombe* homologous to the human thi9maes transporters (ThTr1 and ThTr2, reviewed in Ref. 48), with which it shares 13.7% (ThTr1) or 18.3% (ThTr2) identical amino acids, nor to the thi9maes transporters from *S. cerevisiae* that are 13.1–14.3% identical. The mammalian transporters are related to the carrier for reduced folate, whereas the yeast transporters belong to the family of nucleobasectomy symporters (NCS1). Both of these groups are distantly related to the major facilitator superfamly.

The *S. pombe* Thi9 protein is predicted to have 12 transmembrane domains (Fig. 2A), a number shared with its orthologs and frequently found in the APC superfamly (47). The N- and C-terminal hydrophilic domains of Thi9 are predicted to reside in the cytoplasm. To determine the subcellular localization of Thi9, we constructed a plasmid in which the thi9<sup>+</sup> gene was fused to *gfp*. The plasmid was linearized within the thi9<sup>+</sup> ORF, transformed into *S. pombe* wild type cells, and transformants in which the plasmid had integrated into thi9<sup>+</sup> by homologous recombination were selected (39). In a wild type strain background, Thi9-GFP clearly localized to the cell periphery. Similar to other plasma membrane transporters in *S. pombe* (29, 49), Thi9-GFP accumulated at cell tips and in the septum of dividing cells (Fig. 2B). Thus, Thi9 appears to be a plasma-membrane protein. Wild type cells expressing thi9<sup>+</sup>-gfp were sensitive to pyrithiamine, demonstrating that the tagged protein is functional (Fig. 2C).

ptr1 mutants carry an E81K mutation in the N-terminal region of Thi9 close to the beginning of the first transmembrane domain (Fig. 2A). Our tagging strategy for thi9<sup>+</sup> placed the GFP tag behind the ptr1 mutant allele, enabling the visualization of the Thi9 protein carrying the E81K mutation. We observed an overall decrease in fluorescence and accumulation of Thi9(E81K)-GFP in the endoplasmic reticulum and punctuate cytoplasmic structures (Fig. 2B). This possibly indicates that the protein is subject to quality control mechanisms that prevent its progression in the secretory pathway. A fraction of Thi9(E81K)-GFP was also present in the cell periphery, but it is not clear if this corresponds to the plasma membrane or to cortical ER. Thus, the E81K mutation clearly affects the trafficking of Thi9. However, it cannot be resolved if the pyrithiamine resistance of ptr1 mutants (Fig. 2C) is caused by the mislocalization of Thi9 or if the catalytic activity of Thi9 is additionally affected.

**Bsu1 Contributes to Thiamine Uptake**—To characterize the thiamine transport activity of *S. pombe* cells, we performed uptake assays with [3H]thiamine. All assays were performed in EMM, which has pH 3.8 and contains 2% glucose. Wild type cells had a strong activity in thiamine uptake and this activity was reduced to 25% upon deletion of thi9<sup>+</sup> (Fig. 3A). The remaining activity of thi9Δ cells was sensitive to the protonophore carbonyl cyanide m-chlorophenylhydrazone, indicating that it was caused by a H<sup>+</sup>-symporter (data not shown). We have previously characterized Bsu1 as a plasma-membrane H<sup>+</sup>-symporter for pyridoxine whose gene is regulated by pyridoxine and thiamine (29). To assess if thiamine transport in thi9Δ cells was caused by Bsu1, the uptake assays were repeated with thi9Δ bsu1Δ cells. The double mutants were completely unable to take up thiamine from the extracellular medium (Fig. 3A). Whereas cells containing only Bsu1 (bsu1Δ) possessed 25% of the thiamine transport activity of wild type cells, cells containing only Thi9 (bsu1Δ thi9Δ) had 56% remaining activity. We also performed growth assays with *S. cerevisiae* CVY4 mutants expressing thi9<sup>+</sup> or bsu1<sup>+</sup> from multicycopy plasmids (Fig. 3B). Consistent with the uptake assays, expression of thi9<sup>+</sup> caused much better growth on thiamine-deficient media than expression of bsu1<sup>+</sup>. Expression of either bsu1<sup>+</sup> or thi9<sup>+</sup> improved the growth relative to vector controls, demonstrating that both proteins are active in thiamine uptake (Fig. 3B).

Because these data indicated that the contribution to thiamine uptake of Bsu1 is smaller than that of Thi9 and because we only found thi9<sup>+</sup> in the genetic complementation of thiamine transport-deficient mutants, we focused on a detailed kinetic characterization of Thi9. Experiments to test the thiamine transport activity of Bsu1 are in progress.

**Kinetic Characterization of Thi9**—All uptake assays were performed with *S. pombe* cells containing thi9<sup>+</sup> as the only functional thiamine transporter. First, we analyzed if thiamine uptake via Thi9 was saturable. The apparent *K<sub>m</sub>* value was

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* M. Saier, personal communication.
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A

B

C

wt  wt + thiamine  thi9^{-} - GFP  ptr1  thi9^{-}

10 \mu m  10 \mu m
The pyridoxine transporter Bsu1 contributes to thiamine uptake in S. pombe. A, standard uptake assays were performed with S. pombe cells of the indicated genotype using 2 μM thiamine as a substrate. Uptake velocities were determined with the standard assay in the 2-min time course experiments. B, growth of S. cerevisiae CV4 (thiΔ thi7Δ thi72Δ thi4Δ) compared to a strain harboring the cDNA of S. pombe thi9+, or a p426-GPD plasmid containing bsu1+. The plates were scanned 3 days after plating.

![Graph showing thiamine uptake](image)

FIGURE 3. The pyridoxine transporter Bsu1 contributes to thiamine uptake in S. pombe. A, standard uptake assays were performed with S. pombe cells of the indicated genotype using 2 μM thiamine as a substrate. Uptake velocities were determined with the standard assay in the 2-min time course experiments. B, growth of S. cerevisiae CV4 (thiΔ thi7Δ thi72Δ thi4Δ) compared to a strain harboring the cDNA of S. pombe thi9+, or a p426-GPD plasmid containing bsu1+. The plates were scanned 3 days after plating.

![Graph showing thiamine uptake](image)

FIGURE 4. Thiamine transport activity of S. pombe Thi9. Transport assays were performed with S. pombe bsu1Δ mutant in EMM at 30 °C. After adding a mixture of [3H]thiamine and unlabeled thiamine with a total concentration of 2 μM, aliquots of the cells were filtered and the cell bound radioactivity was determined. Uptake velocities are the mean ± S.D. of three independent determinations. A, thiamine transport assays were performed in the presence of various concentrations of thiamine and uptake velocities were determined from time courses. The graph represents the Lineweaver-Burk plot of one experiment from which the Km value was determined to be 0.4 ± 0.2 μM (mean ± S.D.). B, thiamine transport assays in the presence of the protonophores carbonyl cyanide m-chlorophenylhydrazone (CCCP, 100 μM) and carbonyl cyanide p-(trifluromethoxy)phenylhydrazone (FCCP, 100 μM) or in the absence of glucose. The transport activity in the control experiment was 36.3 ± 3.7 pmol of thiamine × OD cells⁻¹ × min⁻¹. C, thiamine transport assays in the presence of a 10-fold excess (20 μM) of unlabeled thiamine, pyrithiamine, amyloumine, amphilir, HET, or HMP. The transport activity in the control experiment without competitors was 36.1 ± 4.3 pmol of thiamine × OD cells⁻¹ × min⁻¹. D, growth assays with thiamine-depleted S. pombe strains of the given genotype. The cells were plated for a lawn and a filter disc impregnated with 20 μL of a 1.2 μM solution of HET was added. Growth was recorded 2 days after plating. In a similar assay, HMP did not produce any growth. E, thiamine transport assays were performed in the presence of a 10-fold excess (20 μM) of the given compounds. The transport activity in the absence of competitors was 36.1 ± 4.3 pmol of thiamine × OD cells⁻¹ × min⁻¹.

![Graph showing thiamine uptake](image)

FIGURE 2. Pyrithiamine resistance in S. pombe is caused by a mutation in thi9+. A, alignment of Thi9 from S. pombe (Sp, SPAC9.10) with homologous proteins from S. japonicus (Sj, SJAG_04673.1, 67% identical), A. fumigatus (Af, XP_74806, 50% identical), C. neoformans (Cn, XP_774758, 45% identical), and B. fuchiliana (BF, XP_001560408, 50% identical). These organisms do not possess equally well conserved orthologs of the S. cerevisiae thiamine transporter Thi7. In the S. pombe sequence, the 12 transmembrane helices as predicted by Phobius (74) are marked and numbered and the amino acid exchange (E81K) that determines the transporter Thi7. These mutations are also present in C. neoformans, C. albicans, and B. fuchiliana but are not present in A. fumigatus. B, S. pombe wild type cells (left image) or ptr1 mutants (right image) expressing a genomically integrated gfp fusion to the 3’-end of thi9. The cells were grown in thiamine-free EMM and observed by confocal microscopy (Zeiss LSM 510 META). The contrast of the right image was enhanced to allow visualization of the much weaker fluorescence of the ptr1 mutants. C, S. pombe strains of the indicated genotype were grown in EMM without thiamine. D, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. E, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. F, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. G, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. H, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. I, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. J, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. K, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. L, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. M, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. N, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. O, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. P, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. Q, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. R, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. S, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. T, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. U, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. V, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. W, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. X, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. Y, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine. Z, S. pombe strains of the indicated genotype were grown in EMM containing 2 μM thiamine.

![Graph showing thiamine uptake](image)
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propyl-5-pyrimidinyl)methyl]-2-picolinium hydrochloride), another structural analog of thiamine, had a less drastic effect than pyrithiamine and oxythiamine. Of the two structural moieties of thiamine, HET significantly reduced thiamine uptake, whereas HMP had only a minor effect (Fig. 4C). Using nmt2Δ mutants, which are unable to assemble the thiazole part of thiamine, we analyzed if HET only binds to or if it is transported by Thi9. Whereas nmt2Δ bsu1Δ double mutants grew readily around a HET-impregnated filter disc, nmt2Δ bsu1Δ thi9Δ triple mutants showed a much smaller zone of growth (Fig. 4D). In a similar experiment, HMP did not produce any growth (data not shown). Together, this demonstrates that HET is a substrate that is transported by Thi9.

Because the primary structure of Thi9 is related to transporters for positively charged organocations such as basic amino acids, γ-aminobutyric acid, polyamines, and choline, we tested these compounds as competitors. However, none of these substances had a drastic effect on thiamine uptake in S. pombe (Fig. 4E). In addition, thiamine uptake experiments were performed in the presence of pyridoxine, 4-deoxypyridoxine, or amiloride. These substances of the S. pombe pyridoxine transporter Bsu1 were without effect on thiamine uptake (Fig. 4E). We also tested folate, the substrate of the human reduced folate transporter RFC1, which is ~35% identical with the human thiamine transporters ThTr1 and ThTr2. RFC1 is known to also transport thiamine monophosphate (51). However, folate was without effect on thiamine uptake via Thi9 as was the unrelated organocation tetraethylammonium chloride (Fig. 4E).

Regulation of thi9+—Because thiamine is known to regulate the expression of many genes in S. pombe (52–55) and thiamine uptake was earlier found to be reduced in thiamine-sufficient wild type cells (27), we analyzed if the thiamine transporter gene thi9+ is controlled by thiamine. The promoter of thi9+ contains three regions with similarity to a previously identified thiamine-regulatory promoter motif (56) indicating a possible regulation by thiamine. To exhaust the internal thiamine pool, we first cultivated bsu1Δ cells for 3 days in EMM lacking thiamine with daily passages. A parallel culture was grown in thiamine-sufficient (1.2 μM) media. Consistent with results obtained earlier in wild type cells (27), growth of bsu1Δ in the absence of thiamine caused a ~9-fold increase in thiamine uptake (Fig. 5A).

We asked if these differences in thiamine uptake correlated with the expression of thi9+ and how quickly thiamine exerted this effect. For this experiment, thiamine-exhausted wild type cells were shifted to medium containing 1.2 μM thiamine, followed by RNA preparation and analysis by Northern blotting. The thi9+ mRNA was strongly expressed in thiamine-depleted cells, decreased to 34% within 3 min after thiamine addition, and leveled off at 2% of the starting amount at later time points (Fig. 5B). A parallel culture of nmt2Δ mutants was grown for 3 days with severely growth-restricting amounts of thiamine (8 nM), shifted to 1.2 μM thiamine, and used for RNA extraction. Relative to wild type cells, nmt2Δ mutants had increased levels of thi9+ mRNA, but were also subject to thiamine repression (Fig. 5B). Together, these data indicate that the thi9+ promoter is rapidly shut off when thiamine is present and that the transcript has a rapid turnover.

To support the above findings on the regulation of thi9+, we generated strains with tagged versions of thi9+. The Thi9-3HA fusion protein was expressed from integrated alleles under control of the native promoter. In thiamine-exhausted wild type cells, Thi9-3HA was detectable as a 69-kDa protein, which agrees with its calculated molecular mass. The abundance of this protein decreased upon addition of thiamine with a half-life of ~2.5 h, which is similar to the rate at which the cells divide (Fig. 5C). Thus, the Thi9 protein appears to be far more stable than the thi9+ mRNA. The abundance of Thi9-3HA is ~3-fold increased in nmt2Δ mutants. Following the addition of thiamine, it disappears slower than in wild type cells (Fig. 5D), indicating that the synthesis of Thi9 in nmt2Δ is not completely blocked by exogenous thiamine. Consistent with this interpretation, the thi9+ mRNA is more abundant in nmt2Δ mutants when thiamine is present (Fig. 5B).

We also performed Western blots with cells from continuous cultures containing fixed thiamine concentrations. Whereas Thi9-3HA was readily detected in cells from media with up to 0.12 μM, its abundance was drastically reduced at 1.2 μM thiamine (Fig. 5E). Taken together, these results clearly show that thiamine uptake via Thi9 is regulated by the availability of thiamine.

DISCUSSION

Genome sequences are available now for hundreds of organisms and the assignment of gene function is in many cases based on functionally characterized homologous proteins. Although the primary structure of a transport protein may provide useful clues about its function, this feature alone is not sufficient to predict its transported substrate. This is due to the fact that many membrane transport proteins are members of large gene families that contain transporters for unrelated substrates that may even use different transport modes such as uniport, symport, or antiport. The prediction of substrates is also complicated by the fact that only few transporters have been characterized by direct assays and that structural information that would allow modeling of substrate binding sites is only available for a minority of them. This situation calls for increased efforts to characterize transport proteins experimentally and use information from gene expression patterns, genetic or physical interactions, or the analysis of phenotypes caused by gene overexpression or deletion as a guide.

In this work, we establish that two thiamine transporters are encoded in the S. pombe genome. The protein derived from thi9+ is not related to any of the previously known thiamine transporters. It is a distant member of the ACT transporter family within the APC superfamily whose hitherto identified substrates are basic amino acids, γ-aminobutyric acid, polyamines, and choline (TC 2.A.3.4) (47). The thi9+ gene is strongly regulated by the thiamine supply and the protein has a high affinity for its substrate, implying that thiamine transport is indeed the biological function of Thi9. We also found a role for Bsu1 in thiamine uptake in S. pombe. This protein was identified by complementation of S. cerevisiae mutants defective in pyridoxine synthesis and uptake and characterized as a pyridoxine-proton symporter (29). Bsu1 is related to drug exporters from the multidrug resistance family (57) and its gene is regu-
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35.3 ± 4.0 pmol of thiamine × OD cells⁻¹ × min⁻¹ (0 µM thiamine) and 3.8 ± 1.1 pmol of thiamine × OD cells⁻¹ × min⁻¹ (1.2 µM thiamine). Error bars were omitted when smaller than the symbols. B, S. pombe wild type cells and nmt2Δ mutants were pre-cultured for 3 days in media without or with 8 nM thiamine, respectively. The cells were shifted to EMM containing 1.2 µM thiamine and timed aliquots were removed for RNA preparations. The RNA (10 µg/lane) was separated on a formaldehyde gel, transferred to a nitrocellulose membrane, and probed with a radiolabeled DNA fragment spanning the whole coding region of thi9⁻. The signals were quantified on a phosphorimager and corrected for unequal loading using an actin⁻¹ (actin) probe. C, Western blots were performed for wild type cells expressing an integrated version of thi9⁻·3HA under control of its native promoter. The cells were precultured as in B and then shifted to media containing 1.2 µM thiamine. Aliquots of the cells were removed, lysed with glass beads, and a membrane pellet was prepared by centrifugation at 20,000 × g for 10 min. The upper portion shows a Western analysis with a monoclonal antibody directed against the tag. The lower panel shows a Coomassie-stained gel of the same protein preparations. Quantifications of band intensities were performed with the Multi Analyst software and are based on a weaker exposure of the blot. D, Western blots were performed for nmt2Δ mutants expressing an integrated version of thi9⁻·3HA under control of its native promoter. The samples were taken and prepared as in C, quantifications of band intensities are relative to the 100% value of C and are based on a weaker exposure. E, wild type expressing thi9⁻·3HA were continuously grown in media with the indicated thiamine concentrations and extracted as described in C.
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The fact that S. pombe and S. cerevisiae use unrelated proteins for thiamine uptake suggests that these transporters have evolved after the separation of both lines more than 330 million years ago (58). Once efficient plasma membrane transporters were available, environments with a reliable supply of thiamine supported the development of auxotrophic species. Because vitamins are synthesized by multistep enzymatic pathways and are needed in trace amounts only, it is speculated that the loss of their biosynthesis pathways might have provided a selective advantage (59). Indeed, S. cerevisiae and S. pombe grow significantly faster in the presence of exogenous thiamine (46, 52), demonstrating that the synthesis of thiamine is a costly endeavor. In the genus Saccharomyces, only a few non-sensu strictu species such as Saccharomyces servazii, Saccharomyces castellii, and Saccharomyces unisporus require thiamine. In these organisms, thiamine auxotrophy correlates with a lack of orthologs of THI5 and can be complemented with HMP (25). Thiamine auxotrophic species are more frequent in the genera Rhodotorula and Cryptococcus and include the fatal human pathogen Cryptococcus neoformans (4, 60). C. neoformans contains a protein with high similarity to Thi9 from S. pombe (Fig. 2A) and compounds targeting this protein might have antifungal activity.

Prokaryotes appear to possess thiamine transporters that are not related to those of S. cerevisiae or S. pombe. The bacterial transporters are either multisubunit ABC transporters (ThiBPQ) (18) or proteins with six predicted membrane spans and an unknown catalytic mechanism (YuaJ) (11). Neither prokaryotic nor the yeast proteins are related to the mammalian thiamine transporters. ThTr1 was cloned as the gene responsible for thiamine-responsive megaloblastic anemia syndrome (19, 21, 22). The protein likely operates as a high-affinity (K_m = 2.5 μM) thiamine/H^+ antiporter with narrow substrate specificity and is present in many tissues (19, 21, 22, 61). However, its lack does not cause beriberi, likely because of the presence of a second thiamine transporter with overlapping tissue distribution (20, 48). The absence of a phylogenetic relationship between the thiamine transporters known to date, which appear to belong to different transport protein families, suggests that thiamine transporters have evolved several times convergently.

In addition to its role as an enzymatic cofactor, thiamine also influences the expression of genes involved in thiamine biosynthesis and transport and causes the repression of more than 60 genes in S. cerevisiae, 20 of which are repressed >10-fold (6, 7). Unlike most biosynthetic genes, the thiamine transporter gene THI7 does not depend on Thi2 for its regulation (7, 15). This difference could reflect that efficient thiamine uptake requires expression of the thiamine transporter in low-thiamine media, whereas the thiamine biosynthetic genes can be fully repressed under these conditions. In S. pombe, the thiamine biosynthetic gene nmt1^+ is under control of two related transcription factors, Thi1 and Thi5, both of which are necessary for its full expression in media lacking thiamine (62–64). It is known that Thi1 is necessary for the expression of bsu1^+ (55) but the influence of Thi1 and Thi5 on thi9^+ awaits experimental clarification. thi9^+ contains promoter motifs that are also present in other thiamine-regulated genes and are the likely targets of these transcription factors (56). Both mammalian thiamine transporters also appear to be controlled by exogenous thiamine. This is demonstrated at the level of promoter activity, mRNA and protein abundance, which are ≈2-fold increased by thiamine deficiency (65, 66). Modulators of the Ca^{2+}-calmodulin-mediated signaling pathway cause an overall reduction of the thiamine transport activity, but it was not tested if this pathway is also involved in the increased expression of ThTr1 and ThTr2 when thiamine is scarce (65, 66).

In S. cerevisiae and S. pombe, the thiamine biosynthetic genes are fully repressed by exogenous thiamine and strongly expressed in its absence (46, 52, 54, 67, 68). Expression vectors containing the promoter of nmt1^+ are the most frequently employed plasmids for gene expression studies in S. pombe (38, 39, 69–72). A distinct disadvantage of constructs based on the nmt1^+ promoter is their slow induction in thiamine-free media, which takes at least 10 h (28, 52), presumably reflecting the exhaustion of the intracellular thiamine or TDP supply (39, 52). Thiamine transport mutants may hold promise for improving this popular expression system by reducing the time required for the activation of the nmt1^+ promoter.

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