

Amiloride Uptake and Toxicity in Fission Yeast Are Caused by the Pyridoxine Transporter Encoded by *bsu1*⁺ (*car1*⁺)

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Amiloride, a diuretic drug that acts by inhibition of various sodium transporters, is toxic to the fission yeast *Schizosaccharomyces pombe*. Previous work has established that amiloride sensitivity is caused by expression of *car1*⁺, which encodes a protein with similarity to plasma membrane drug/proton antiporters from the multi-drug resistance family. Here we isolated *car1*⁺ by complementation of *Saccharomyces cerevisiae* mutants that are deficient in pyridoxine biosynthesis and uptake. Our data show that Car1p represents a new high-affinity, plasma membrane-localized import carrier for pyridoxine, pyridoxal, and pyridoxamine. We therefore propose the gene name *bsu1*⁺ (for vitamin B₆ uptake) to replace *car1*⁺. Bsu1p displays an acidic pH optimum and is inhibited by various protonophores, demonstrating that the protein works as a proton symporter. The expression of *bsu1*⁺ is associated with amiloride sensitivity and pyridoxine uptake in both *S. cerevisiae* and *S. pombe* cells. Moreover, amiloride acts as a competitor of pyridoxine uptake, demonstrating that both compounds are substrates of Bsu1p. Taken together, our data show that *S. pombe* and *S. cerevisiae* possess unrelated plasma membrane pyridoxine transporters. The *S. pombe* protein may be structurally related to the unknown human pyridoxine transporter, which is also inhibited by amiloride.

Vitamin B₆ is a generic term describing a group of three water-soluble vitamins, pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM), and the 5'-phosphates derived therefrom. Vitamin B₆ is synthesized in plants and most microorganisms but cannot be produced by mammals and thus represents an essential component of the human diet.

Vitamin B₆ has a central role in the metabolism of amino acids. PL phosphate (PLP) is the cofactor of amino acid transaminases and decarboxylases and enzymes involved in elimination and replacement reactions (19). A more recently discovered function of vitamin B₆ is protection from reactive oxygen species: PN and PLP are as effective as the well-established antioxidant vitamins C and E in quenching singlet oxygen species (6, 10). Moreover, vitamin B₆ is an intermediate in the synthesis of thiamine (vitamin B₁). This metabolic pathway is best known for *S. cerevisiae* cells, where the pyrimidine moiety of thiamine derives from histidine and PN (reference 49 and references therein). The *S. cerevisiae* *THI5* and *THI20* gene families are involved in this pathway (44), and both families have orthologues in many other yeast species. Thus, the yeast thiamine biosynthetic pathway is different from the pathway found in many prokaryotes, where PN is not used as a precursor (43). In addition to having evolved different strategies to synthesize the pyrimidine unit of thiamine, synthesis of the thiazole moiety also follows different routes in pro- and eukaryotes (43).

To date, two different pathways for PN biosynthesis have been described. The two pathways differ in the origin of the ring nitrogen of PN that is derived from the amide group of glutamine in *S. cerevisiae* (39) and from glutamate in *Escherichia coli* (21). The two pathways also utilize different enzymes

that are encoded by *pdxA* and *pdxJ* in *E. coli* and by the *SNZ* (*SOR1/pyroA/PDX1/pdxS*) and *SNO* (*PDX2/pdxT/pyroB*) genes in organisms that lack *pdxA* and *pdxJ* orthologues (24). The three pairs of *SNZ-SNO* genes present in *S. cerevisiae* have a divergent orientation and have a promoter in common, allowing coordinated synthesis of the proteins, which are known to form a multimeric complex (5, 9, 30, 32). Snz2p and Snz3p are almost identical, as are Sno2p and Sno3p (30). Whereas expression of *SNZ2-SNO2* and *SNZ3-SNO3* is repressed by thiamine, the *SNZ1-SNO1* genes do not respond to the availability of thiamine (32).

Filamentous fungi, as well as the fission yeast *S. pombe*, possess only a single copy of an *SNZ* and an *SNO* gene. These genes are linked in *Neurospora crassa* (4), but they are nonadjacent in the plant pathogenic fungus *Cercospora nicotianae* (11) and in *S. pombe* (45). It was discovered recently that Sno-like proteins have enzymatic activities as glutaminases (5, 9). This supports a mechanism by which the ammonium ion released by Sno1p is channeled to Snz1p in the Sno-Snz protein complex (3, 5, 9) and then transferred to an unknown reaction intermediate in the PN biosynthetic pathway. These findings explain why Sno activity is dispensable under certain conditions such as when the medium contains high concentrations of ammonia (5) or when *SNZ* is overexpressed (11, 38).

An alternative pathway to PN biosynthesis lies in the utilization of external vitamin B₆ sources. PN uptake in *S. cerevisiae* depends on the presence of the plasma membrane PN transporter encoded by *TPN1* (38). Tpn1p is a high-affinity ($K_m = 0.55 \mu\text{M}$) proton-PN symporter and is related to the yeast purine/cytosine transporter Fcy2p. These two proteins were variously assigned to the major facilitator superfamily (28) or to the family of nucleobase-cation symporters (31) of yeast transport proteins. Consistent with the presence of *TPN1* orthologues in most yeast species, many yeast species can accumulate PN (47). In *S. pombe*, the apparent K_m values for PN

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uptake were 22.4 and 118 μM and PN uptake was sensitive to amiloride (48). Because amiloride affects many Na^+ -transporting proteins (20), this observation has led to speculation that PN uptake in *S. pombe* cells is driven by Na^+ symport (48).

We have not been able to find relatives of Tpn1p in *S. pombe*, suggesting that similar proteins are not involved in PN uptake in fission yeast. This conclusion was also supported by the different K_m values determined for the two activities. We therefore decided to identify the *S. pombe* PN transporter by complementation of *S. cerevisiae* mutants.

In this paper, we report that the *S. pombe* PN transporter is encoded by the *car1*⁺ (changed amiloride resistance) gene. This gene was previously known to cause amiloride sensitivity and to be regulated by thiamine (18, 29). Our study establishes that Car1p is a proton symporter that transports vitamin B₆ and the drug amiloride. Therefore, and to avoid confusion with the *S. pombe* gene encoding arginase, which is also named *car1*⁺ (41), we will refer to the *car1*⁺ gene as *bsu1*⁺ (for vitamin B₆ uptake).

MATERIALS AND METHODS

Yeast strains, transformations, and media. *S. pombe* strains FY254 (*ade6-M210 can1-1 leu1-32 ura4-D18 h*) (22) and *leu1.32 (leu1-32 h⁻)* (14a) were used in this study. *S. pombe* was transformed by standard methods (2). The *S. cerevisiae* strains were derived from W303-1A (*MATa leu2-3,112 his3-11,15 trp1-1 ade2-1 ura3-1 can1-100*) (40) and transformed by previously published methods (1). The complete medium for both yeasts was YPD (2% yeast extract, 1% peptone, 2% glucose). EMM, a medium devoid of thiamine and PN (23), was used as a minimal medium for *S. pombe* cells. When indicated, thiamine and PN were added from filter-sterilized stocks. SD (2% glucose, 0.67% yeast nitrogen base without amino acids [Difco]) was the minimal medium used for *S. cerevisiae*. SD contains 2 μg of PN-HCl per liter (1.93 μM) and 400 μg of thiamine-HCl per liter (1.07 μM). Media with lower concentrations of PN were made from yeast nitrogen base without amino acids without vitamins (Bio 101) and contained all of the vitamins except PN in the standard concentrations present in yeast nitrogen. Only the required nucleobases and amino acids were added to minimal medium.

Gene disruptions and tagging constructs. *S. cerevisiae* strain MVY30 (W303-1A *snz1-sno1 Δ ::his5⁺ tpn1 Δ ::kanMX4*) was generated by transformation of W303-1A *snz1-sno1 Δ ::his5⁺* (38) with a *tpn1 Δ ::kanMX4* disruption cassette that was amplified from a deletion strain (Euroscarf, Frankfurt/Main, Germany). MVY30 is unable to synthesize PN in thiamine-containing medium because the *SNZ2-SNO2* and *SNZ3-SNO3* genes are not expressed (32). Moreover, because *TPN1* is deleted, the strain is unable to actively transport PN and thus requires 2 μM PN for growth.

The genomic copy of *bsu1*⁺ was fused with GFP or a 3HA sequence with integrative vectors (35). To this end, the *bsu1*⁺ cDNA lacking a promoter was ligated with the green fluorescent protein (GFP) or three-hemagglutinin (3HA) sequence (to give C-terminal fusions), followed by an *S. cerevisiae ADH1* terminator in a pBluescript vector. Next, the *S. cerevisiae LEU2* gene was added as a transformation marker downstream of the terminator. After restriction with BglII, which cuts within *bsu1*⁺, the plasmid was transformed into *S. pombe leu1.32* (GFP construct) or FY254 (3HA construct) cells. When integrated by homologous recombination, *bsu1*⁺ is followed by the tag sequences, the *ADH1* terminator, the *LEU2* gene, and a partial duplication of *bsu1*⁺ that lacks a promoter and will not be expressed. Correct transformants were identified by Western blotting or fluorescence microscopy. Expression of a *bsu1*⁺-GFP fusion restored PN uptake in *S. cerevisiae* MVY30 cells, indicating that C-terminal tagging does not interfere with the activity of the protein (data not shown).

To generate a disruption construct for *bsu1*⁺, the cDNA was amplified with PCR primers to eliminate the EcoRI site 68 bp upstream of the stop codon. The PCR product was ligated into the NotI site of a modified pUC19 plasmid and cut at the two remaining EcoRI sites to remove 723 bp from the *bsu1*⁺ coding region. Next, *ura4⁺* was integrated as an EcoRI fragment from pIC20R-*ura4⁺* (37). In the final plasmid, *ura4⁺* was flanked by 126 bp from the 5' end and 611 bp from the 3' end of *bsu1*⁺. The cassette was released with NotI and used to transform FY254 or FY254 *snz1 Δ ::kanMX4* cells to uracil prototrophy.

To disrupt *snz1*⁺ in *S. pombe*, the NotI insert of library plasmid pFL61-*snz1*⁺

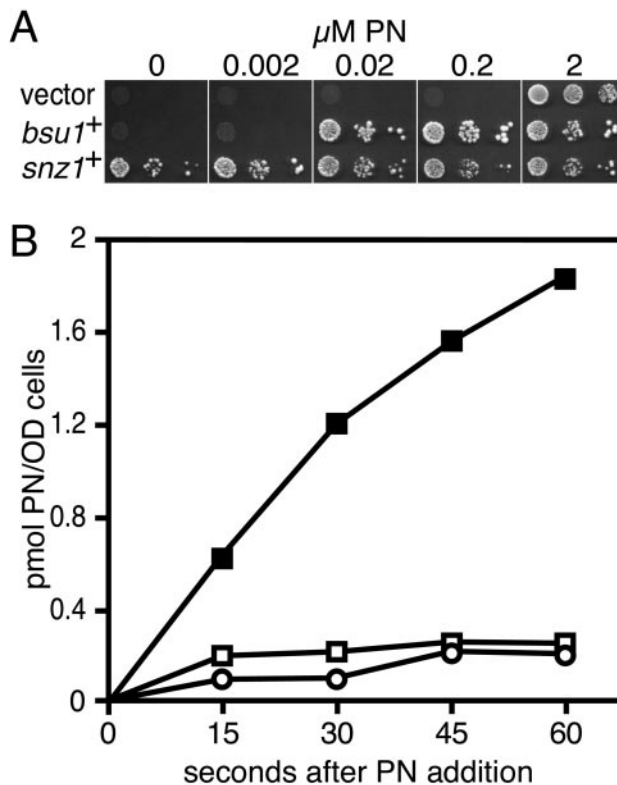


FIG. 1. Characterization of complemented *S. cerevisiae* MVY30 mutants. MVY30 was transformed with an *S. pombe* cDNA library, and transformants that were able to grow on plates containing 0.2 μM PN were selected. These transformants were found to contain either the *snz1*⁺ or the *bsu1*⁺ cDNA. In panel A, the transformants were tested for the ability to grow on various concentrations of PN. MVY30 cells with an empty pFL61 plasmid served as a control. In panel B, PN transport activity was assayed for the same cells with a substrate concentration of 10 μM PN. (○, vector transformants; □, cells expressing *snz1*⁺; ■, cells expressing *bsu1*⁺). OD, optical density.

was ligated into a modified pUC19 plasmid. Next, 330 bp were removed from the *snz1*⁺ coding region with HincII and replaced with an EcoRV-SmaI fragment from pFA6a-kanMX4 (42) containing the G418 resistance gene. In the final construct, *kanMX4* was flanked with 489 bp from the 5' end and 380 bp from the 3' end of *snz1*⁺. The cassette was excised with NotI and transformed into FY254 or FY254 containing a 3HA-tagged *bsu1*⁺ gene, and the cells were selected on YPD containing 200 mg of G418 per liter. All deletions were confirmed by PCR.

Uptake experiments were performed with *S. cerevisiae* MVY30 cells expressing *bsu1*⁺ from pFL61 (see Fig. 1 and 5) (27) or from pTG10249 (see Fig. 3) (26).

Identification of *bsu1*⁺ and *snz1*⁺ by complementation. MVY30 was transformed with an *S. pombe* cDNA library generated in *S. cerevisiae* multicopy plasmid pFL61 (27). In the library plasmids, the cDNAs in the NotI site are flanked by the promoter and terminator sequences of *PGKI*, allowing high-level expression in *S. cerevisiae*. The transformed cells were plated on SD medium containing 0.2 μM PN. Transformants were picked after 3 to 5 days and characterized by growth assays and PN uptake experiments. The plasmids of selected clones were rescued into *E. coli*, and their inserts were sequenced.

Northern and Western blotting. *S. pombe* cells for use in Northern or Western blot analyses were precultured in EMM containing 1 μM PN until they reached stationary phase. They were then washed in EMM without additions and transferred to the indicated medium, where they were grown for at least 12 h.

Northern blots were prepared as described previously, incubated with [³²P]CTP-labeled probes, and quantified with a PhosphorImager (36). The *bsu1*⁺ and *snz1*⁺ probes corresponded to the entire cDNAs taken from library plasmids. Signals obtained with a PCR product corresponding to nucleotides 336 to 1216 of the *S. pombe* actin gene *act1*⁺ were used for normalization. For Western blot assays, 5 A_{600} units of cells were broken with glass beads in 100 μl of 1 \times sodium dodecyl sulfate (SDS) sample buffer containing protease inhibitors and

incubated for 2 min at 50°C, and 5 to 10 μ l (as required for equal loading) was separated per lane. The blot was incubated with rabbit anti-HA serum (sc-805; Santa Cruz Biotechnology), and bands were visualized with Super Signal (Pierce) chemiluminescence reagents.

Other techniques. Uptake experiments were performed as previously described for *S. cerevisiae* (38). Standard assay mixtures contained 10 to 50 μ M PN (a mixture of [3 H]PN [ARC, St. Louis, Mo.] and unlabeled PN), 1% D-glucose, 1 to 10 A_{600} units of cells per ml, and citrate-phosphate buffer at pH 4.5.

Confocal microscopy was performed on a Zeiss LSM 510 META microscope. *S. pombe* cells were grown to logarithmic phase in EMM.

Dilution series of yeast cells for use in growth assays were prepared in a 96-well plate. Cells in the first well were diluted to an A_{600} of 0.8 and then diluted 10-fold in each of the next wells. The cells were transferred to plates with a stainless steel replication device and grown at 30°C for 3 days.

RESULTS

S. pombe genes that complement *S. cerevisiae* PN mutants.

Previous work by our group has identified *TPN1*, an *S. cerevisiae* gene encoding the first known plasma membrane transport protein for vitamin B₆ (38). Tpn1p and proteins with similarities to the related purine/cytosine permease Fcy2p are present in many yeast species, but searches of the completed genomic sequence failed to identify orthologues in *S. pombe*. This was unexpected because *S. pombe* was known to actively transport PN (48).

In order to identify the *S. pombe* PN transporter, we used a genetic approach based on complementation of *S. cerevisiae* strain MVY30. MVY30 has mutations in the PN biosynthetic pathway and lacks *TPN1*. This strain has no detectable PN uptake activity and is unable to grow at external PN concentrations of 0.2 μ M or less (Fig. 1). We transformed MVY30 cells with an *S. pombe* cDNA library and selected for plasmids that restored growth on 0.2 μ M PN. The transformants fell into two different classes. The larger group was able to grow on plates containing no PN, indicating that they were able to synthesize PN (Fig. 1A). These transformants were found to contain cDNAs corresponding to open reading frame SPAC29B12.04. We will refer to SPAC29B12.04 as *snz1*⁺ because the protein it encodes is 80% identical to *S. cerevisiae* Snz1p, a protein involved in PN biosynthesis (7, 12). The isolation of *snz1*⁺ confirms previous results that demonstrated that expression of an *SNZ* gene alone can complement the PN auxotrophy of *S. cerevisiae snz1-sno1* mutants (38).

The second class of transformants was represented by a single clone. This clone was unable to grow in medium devoid of PN but could utilize low PN concentrations that did not support the growth of vector controls (Fig. 1A). Interestingly, these cells possessed high levels of PN uptake activity compared to that of cells expressing *snz1*⁺ or vector controls (Fig. 1B). The cDNA that allowed PN uptake corresponded to open reading frame SPAC17A2.01, also known as *car1*⁺. We will refer to this gene as *bsu1*⁺ (for vitamin B₆ uptake) because the experiments described in this paper provide evidence that it encodes the plasma membrane PN transporter of *S. pombe*. *bsu1*⁺ encodes a protein of 526 amino acids that confers sensitivity to the antihypertensive drug amiloride (18). Similar to other proteins involved in membrane transport, Bsu1p contains multiple hydrophobic domains likely to represent 12 membrane-spanning α -helices (18). Bsu1p is a member of the major facilitator superfamily and is most similar to proteins from the multidrug resistance (MDR) family that function as drug exporters (14). In addition, expression of *bsu1*⁺ is regu-

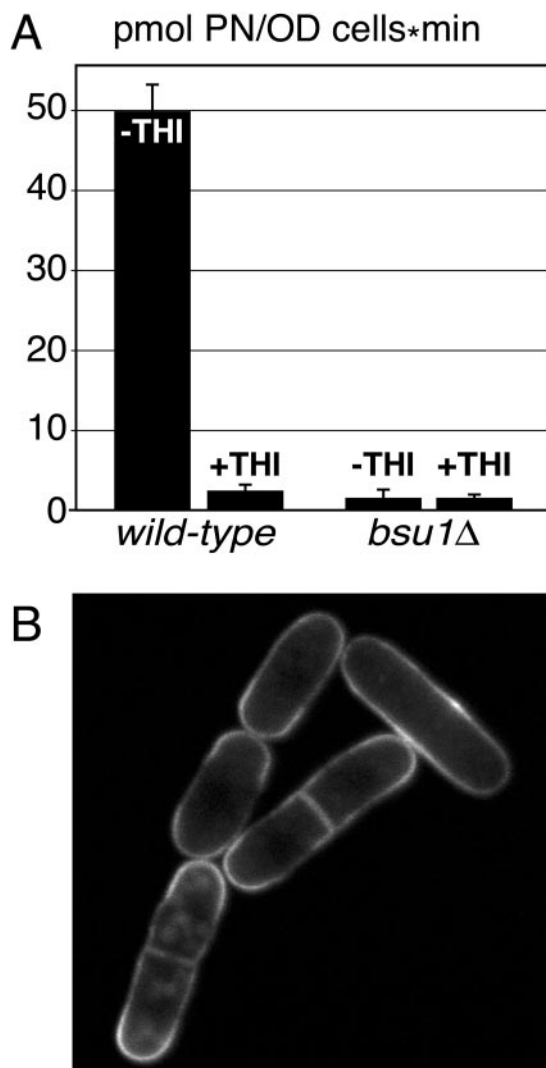


FIG. 2. Effect of thiamine on PN transport and localization of Bsu1p-GFP. In panel A, wild-type or *bsu1* Δ mutant *S. pombe* cells were used in standard PN transport assays with 10 μ M substrate. The cells were grown in EMM (-THI) or in EMM containing 1 μ M thiamine (+THI). Bars represent average values from three independent determinations, and standard deviations are presented as error bars. In panel B, *S. pombe* cells were transformed with an integrative plasmid that tagged the genomic copy of *bsu1*⁺ with GFP. To induce expression of *bsu1*⁺, the cells were grown in thiamine-free EMM and observed by confocal microscopy. OD, optical density.

lated by thiamine, but Bsu1p does not appear to be involved in thiamine transport (29). Thus, although much was known about the interaction of Bsu1p with amiloride and the regulation of the gene, the substrate transported by Bsu1p remained unknown.

***bsu1*⁺ encodes the plasma membrane PN transporter of *S. pombe*.** To characterize the PN transport activity of Bsu1p in more detail, we performed uptake experiments with *S. cerevisiae* MVY30 cells expressing *bsu1*⁺. PN uptake was optimal at pH 4.5 and followed saturation kinetics. The apparent K_m value for PN uptake was determined to be 24 μ M, which is in agreement with the lower of the two K_m values previously reported for *S. pombe* cells (48).

Next we investigated PN transport in wild-type *S. pombe*

cells and in cells carrying a disrupted allele of *bsu1*⁺. Wild-type cells that were grown in the absence of thiamine had high PN uptake activity. In agreement with the regulation of *bsu1*⁺ by thiamine, we found a drastic reduction in PN transport upon growth in thiamine-containing medium (Fig. 2A). PN uptake activity was also reduced by more than 90% upon disruption of *bsu1*⁺ (Fig. 2A), confirming the role of *bsu1*⁺ in PN uptake. Moreover, assuming an intracellular volume of 1 $\mu\text{l}/A_{600}$ unit of cells, PN was accumulated more than fivefold within 1 min in wild-type cells, indicating that PN uptake is an energy-requiring process. Thus, our results are consistent with the notion that Bsu1p is an active plasma membrane transporter for PN.

To provide a more detailed picture of the localization of Bsu1p, we C-terminally tagged the Bsu1 protein with GFP by integration of a fused gene at the *bsu1*⁺ locus. When *S. pombe* cells expressing Bsu1p-GFP were observed by confocal microscopy, all of the cells showed fluorescence at the plasma membrane (Fig. 2B). In many of the cells, the fluorescence was stronger in the cell tip than in the rest of the cell (Fig. 2B). In more elongated cells, an increase in fluorescence was observed around the middle of the cell. In cells that underwent mitosis, Bsu1p-GFP additionally appeared at the septum. A similar distribution resulted from expression of *bsu1*⁺-GFP from a plasmid under the control of a strong promoter, and no fluorescence was observed when 10 μM thiamine was present during growth (data not shown). In summary, our experiments provide evidence that the physiological role of Bsu1p in *S. pombe* is that of a thiamine-regulated PN transporter operating at the plasma membrane.

Bsu1p is capable of transporting PN, PL, and PM. We performed growth and uptake experiments with MVY30 cells to analyze the range of substrates that can be transported by Bsu1p (Fig. 3). MVY30 cells transformed with a control vector were unable to grow on plates containing less than 2 μM PN or PL, and no growth occurred on plates containing PM. In contrast, *bsu1*⁺-expressing cells required 0.02 μM PN or PL for growth (Fig. 3A). PN showed higher potency than PL in supporting the growth of *bsu1*⁺-expressing cells, and growth also occurred on 2 μM PM. Neither strain was capable of growing on plates devoid of vitamin B₆, indicating that the growth differences did not derive from intracellular vitamin B₆ carried over with the inoculum.

The same substrate preferences were apparent when PL and PM were tested as competitors of PN uptake (Fig. 3B). Whereas a 10-fold molar excess of PM reduced PN uptake by only 10%, PN transport was reduced by 65% in the presence of PL. As in *S. cerevisiae* (38), 4-deoxypyridoxine was the most potent competitor and reduced PN uptake even more effectively than did unlabeled PN (Fig. 3B).

We also tested PN uptake in the presence of uncouplers of the transmembrane proton gradient. As shown in Fig. 3C, addition of the protonophore carbonyl cyanide *m*-chlorophenylhydrazine, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazine, or 2,4-dinitrophenol (16) drastically reduced PN uptake via Bsu1p, and the remaining activity amounted to 15 to 30% of that of the untreated control cells. In summary, Bsu1p is able to transport PN, PL, and PM and acts as a proton symporter.

Expression of *bsu1*⁺ responds to thiamine and PN. We have shown above that cells from thiamine-containing medium possessed reduced PN uptake activity (Fig. 2A). This confirmed

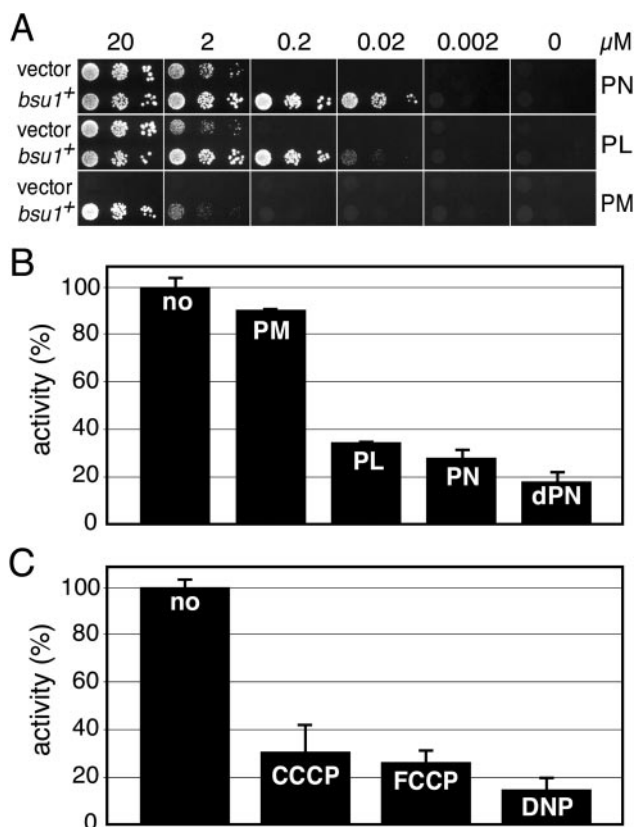


FIG. 3. Substrate specificity of Bsu1p. *S. cerevisiae* MVY30 cells expressing *bsu1*⁺ or harboring a control plasmid were tested for the ability to transport different forms or analogs of vitamin B₆. In panel A, the results of growth assays on plates containing PN, PL, or PM are presented. In panel B, uptake assays were performed in the absence or presence of the indicated competitors. Labeled PN was present at a concentration of 10 μM , and competitors were added in 10-fold excess relative to PN. A value of 100% corresponds to an uptake activity of 53.0 pmol of PN per A_{600} unit of cells per min. In panel C, the uncoupler CCCP (100 μM), FCCP (100 μM), or DNP (5 mM) was present during an uptake assay performed with 10 μM substrate. A value of 100% corresponds to an activity of 72.8 pmol of PN per A_{600} unit of cells per min. The uptake assays in panels B and C were performed in triplicate. Bars represent average values for the PN uptake velocity, and error bars represent the standard deviation. dPN, 4-deoxypyridoxine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazine; DNP, 2,4-dinitrophenol.

previous findings on the regulation of *bsu1*⁺ by thiamine (29) and raised the question of whether *bsu1*⁺ is also regulated by PN.

The *bsu1*⁺ mRNA was undetectable in thiamine-grown wild-type cells, and this was not influenced by the presence or absence of PN (Fig. 4A). In contrast, *bsu1*⁺ was expressed when wild-type cells were grown in medium lacking thiamine but the relative abundance of *bsu1*⁺ mRNA changed very little with the amount of PN present (Fig. 4A). In PN auxotrophic *snz1* Δ mutants, however, the abundance of the *bsu1*⁺ mRNA increased when cells were grown in PN-deficient medium (Fig. 4B). After normalization, we found that *bsu1*⁺ expression was induced threefold in cells grown in the absence of PN relative to that in cells grown in medium with sufficient PN (Fig. 4B).

To analyze if the changes in the *bsu1*⁺ mRNA levels are also

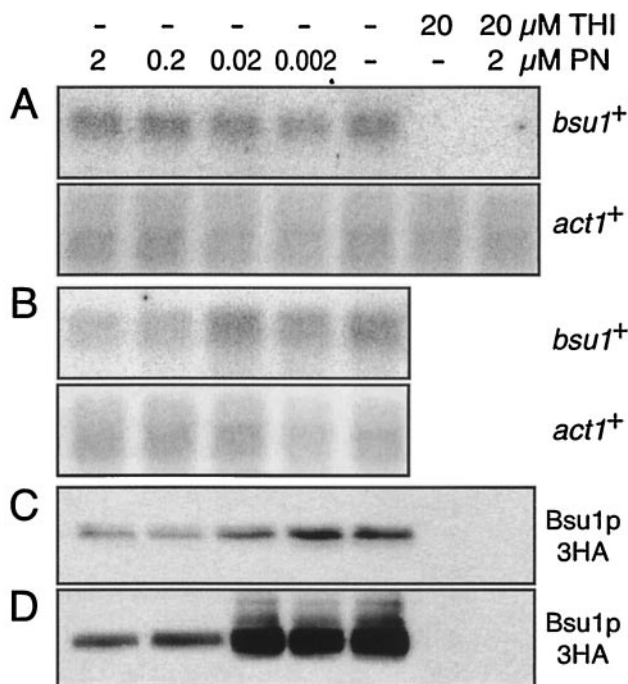


FIG. 4. Influence of thiamine and PN on the expression of *bsu1*⁺. Cells were grown in the concentrations of thiamine (THI) or PN shown and used for the determination of *bsu1*⁺ expression by Northern analysis (A and B) or Western blotting (C and D). Wild-type cells were used in panel A, and *snz1*Δ mutant cells were used in panel B for RNA preparations. The RNA was separated, blotted onto nitrocellulose membranes, and hybridized to ³²P-labeled probes to detect *bsu1*⁺ or *act1*⁺. In panels C and D, total protein extracts were prepared from cells carrying a 3HA-tagged version of *bsu1*⁺ in a wild-type (C) or *snz1*Δ mutant (D) background. Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and detected with a polyclonal serum directed against the HA tag. In panels C and D, equal loading was confirmed by SDS-polyacrylamide gel electrophoresis, followed by Coomassie staining (data not shown).

reflected at the level of the protein, we tagged the genomic copy of *bsu1*⁺ by addition of a 3HA sequence to enable detection of the fusion protein. Tagging of Bsu1p resulted in a 60-kDa protein (calculated molecular mass of Bsu1p-3HA, 62.5 kDa) that appeared in tagged strains but was absent from wild-type controls (data not shown). Growth of Bsu1p-3HA cells in medium containing high concentrations of PN resulted in only weak signals, the strength of which increased slightly toward lower concentrations of PN (Fig. 4C). When the same experiments were performed with a PN auxotrophic *snz1*Δ mutant strain, the amount of Bsu1p-3HA was much greater at each of the PN concentrations analyzed (Fig. 4D). Moreover, the amount of Bsu1p-3HA increased dramatically when the medium contained less than 0.2 μM PN (Fig. 4D). In agreement with the results of the Northern blot assays (Fig. 4A and B) and uptake experiments (Fig. 2A), Bsu1p-3HA was undetectable when the medium contained thiamine. Together, these findings show that expression of *bsu1*⁺ is regulated by the availability of PN and by thiamine.

Expression of *bsu1*⁺ causes amiloride sensitivity. The data presented above established that Bsu1p is a plasma membrane-localized transporter of vitamin B₆ in *S. pombe*. What remained unclear was why mutants lacking *bsu1*⁺ were originally

identified by their resistance to the drug amiloride. In *S. pombe* (48) and in human cell lines (33), PN uptake is drastically reduced in the presence of amiloride. Moreover, amiloride and PN are structurally related molecules (Fig. 5A), making it possible that both compounds are imported into *S. pombe* cells via Bsu1p. This hypothesis was tested by uptake experiments performed with 5 or 10 μM PN in the presence of various concentrations of amiloride. The data, presented in the form of a Dixon plot (Fig. 5B), clearly demonstrate that amiloride is a competitive inhibitor of PN transport, with an effective *K_i* of 1.1 μM.

The interaction of amiloride with Bsu1p was further studied by assays of growth on amiloride-containing plates (Fig. 5C). In the absence of amiloride, we detected no differences in the growth of *bsu1*Δ mutant and wild-type cells. Consistent with the absence of *snz1*⁺ paralogues in *S. pombe*, *snz1*Δ mutants showed a strict auxotrophy for PN. The PN requirement of *snz1*Δ cells could not be met by thiamine, indicating that thiamine cannot provide PN in *S. pombe*. *bsu1*Δ *snz1*Δ double mutants were similar to *snz1*Δ single mutants, indicating that, as in *S. cerevisiae* (Fig. 3A), PN can enter cells by unspecific processes when present at an external concentration of 2 μM.

When the same strains were tested on plates containing 200 μM amiloride (Fig. 5C), wild-type cells were sensitive to amiloride in the absence of thiamine, but the growth inhibition caused by amiloride was released when thiamine was present. In contrast, *bsu1*Δ mutant cells were unaffected by amiloride and possessed full growth independent of the addition of PN, thiamine, or both. *snz1*Δ mutants behaved like wild-type cells and were amiloride sensitive without thiamine but resistant in the presence of thiamine. Thus, amiloride resistance in *S. pombe* results from lack of expression of *bsu1*⁺, which can be conferred by deletion of *bsu1*⁺ or by addition of thiamine.

S. cerevisiae cells do not contain a gene with high similarity to *bsu1*⁺, and PN is taken up via the unrelated transporter Tpn1p. We wondered whether *S. cerevisiae* is sensitive to amiloride and found no effect of amiloride on the growth of wild-type cells (Fig. 5D). Expression of *bsu1*⁺ in *S. cerevisiae*, in contrast, caused amiloride sensitivity, showing that wild-type *S. cerevisiae* cells are resistant because they lack amiloride uptake.

DISCUSSION

***bsu1*⁺ encodes a novel PN transporter.** To ensure a sufficient supply of vitamin B₆, yeast cells possess two pathways that lead to the acquisition of intracellular vitamin B₆. One pathway is de novo synthesis of vitamin B₆, and the other pathway depends on plasma membrane transport proteins that mediate vitamin uptake from the environment.

We recently identified the *S. cerevisiae* plasma membrane PN transporter Tpn1p (38). Proteins with similarity to Tpn1p are present in many yeast species, but sequence comparisons showed that *S. pombe* does not possess an obvious Tpn1p homologue. In this report, we provide evidence that the PN transporter of *S. pombe* is encoded by the unrelated gene *bsu1*⁺. This is demonstrated by the fact that in wild-type *S. pombe* cells and *S. cerevisiae* cells expressing *bsu1*⁺, PN transport has similar apparent *K_m* values, has the same optimum pH, and is inhibited by amiloride. Compared with Tpn1p, which has an apparent *K_m* value of 0.55 μM, Bsu1p has a lower affinity for its substrate, PN. Both proteins, in contrast, have

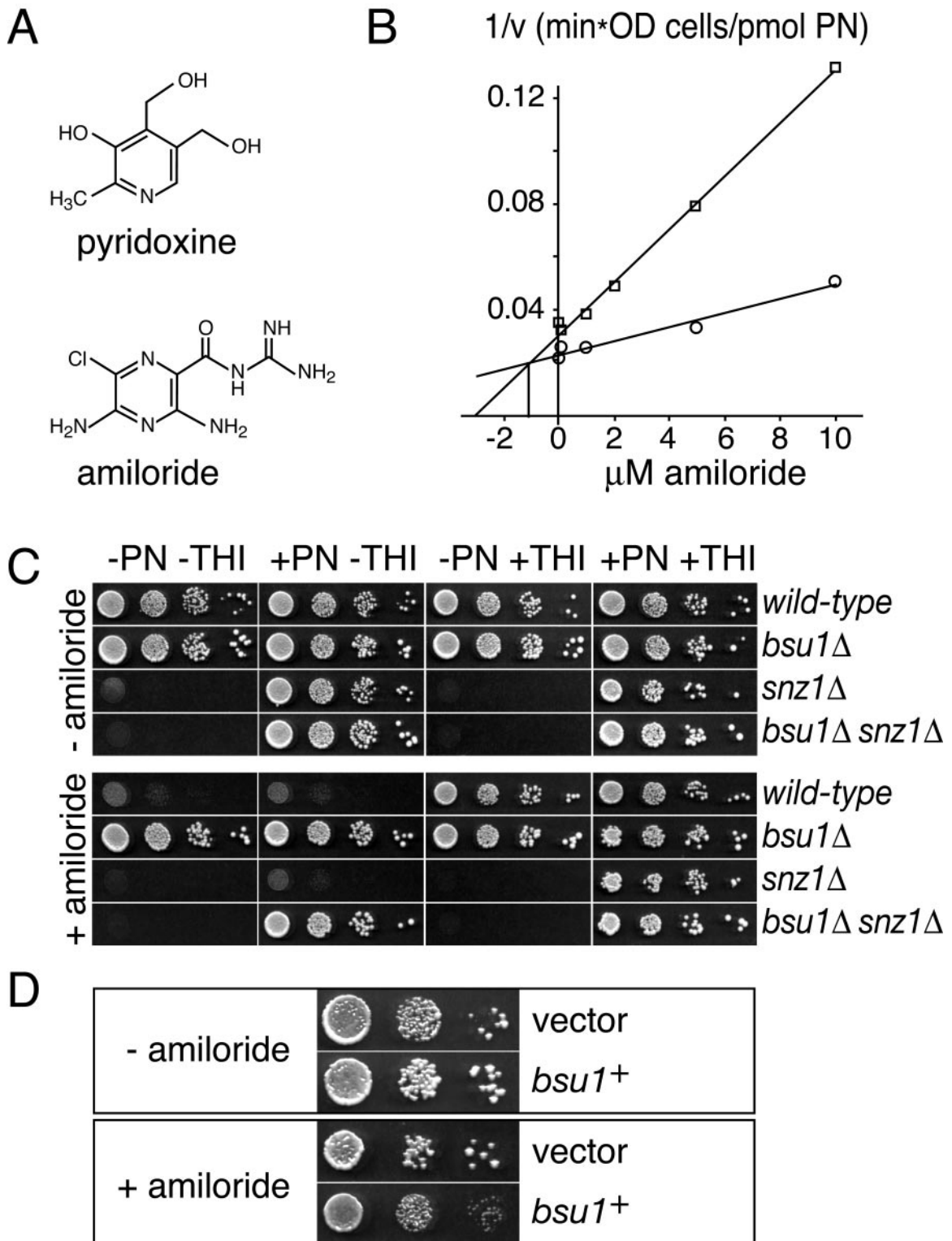


FIG. 5. Interaction of amiloride with the PN transporter Bsu1p. (A) Comparison of the structures of amiloride and PN. Note that the structures shown are those of the neutral compounds. Amiloride is a weak base and positively charged at pH <8.8. PN is positively charged at pH <5.0, negatively charged at pH >8.96, and neutral between pHs 5.0 and 8.96. (B) Amiloride was used as a competitor in PN uptake experiments performed with MVY30 cells expressing *bsu1*⁺. The substrate concentrations were 5 (\square) and 10 (\circ) μM PN. The data, presented in the form of a Dixon plot, allowed the determination of the K_i of amiloride, which is 1.1 μM (dashed line). (C) Growth assays were performed with wild-type *S. pombe* cells or with strains carrying deletions in *snz1*⁺, *bsu1*⁺, or both. EMM plates were supplemented with 2 μM PN (+PN), 1 μM thiamine (+THI), or 200 μM amiloride (+ amiloride) as indicated. (D) Wild-type *S. cerevisiae* W303-1A cells were transformed with pFL61 (vector control) or with pFL61-*bsu1*⁺ and assayed for growth on SD plates containing no or 200 μM amiloride. OD, optical density.

similar optimum pHs and identical substrate profiles and operate as PN-proton symporters, a conclusion supported by the inhibition by protonophores (Fig. 3C) (38, 48).

Database searches with Bsu1p as a query sequence identified two homologous *S. pombe* proteins encoded by SPCC576.17c (44% identical) and SPCC965.13 (43% identical). It is not clear if these proteins are responsible for the residual PN uptake activity observed after deletion of *bsu1*⁺ or growth in thiamine (Fig. 2A). Yagi et al. (48) postulated the existence of a high-affinity system, which we believe to be encoded by *bsu1*⁺, and of a low-affinity system ($K_m = 118 \mu\text{M}$) that might be encoded by these *bsu1*⁺ paralogs. The same group also reported the presence of a PN-exporting system (17), a function that could also be performed by the paralogous proteins.

Bsu1p-related proteins include the *S. cerevisiae* MDR drug antiporters Sge1p, Flr1p, Qdr1p, Tpo1p, and Dtr1p. These proteins are 25 to 27% identical to Bsu1p and catalyze the export of compounds as diverse as crystal violet, ethidium bromide, and other cationic dyes (Sge1p), diazaborine and fluconazole (Flr1p), ketoconazole, fluconazole and quinidine (Qdr1p), spermidine (Tpo1p), and dityrosine (Dtr1p) (14). Bsu1p is also distantly related to Hol1p, an MDR protein that has no assigned function in drug export. Mutations in *HOLI* that result in single amino acid changes allow the protein to act as an import carrier for histidinol and other cations (13, 46). Thus, Bsu1p and Hol1p are exceptional in that their primary structure resembles that of export carriers whereas their catalytic function lies in the import of substrates.

Bsu1p is not involved in amiloride export. Because of the similarity of Bsu1p to the proteins from the MDR family, Bsu1p was repeatedly assigned a function in amiloride export (8, 14). However, our data (Fig. 5) and the data of others (18, 29) are consistent only with Bsu1p mediating the entry of amiloride. We show that PN and amiloride compete for the transport mediated by Bsu1p (Fig. 5B) and demonstrate that *S. cerevisiae*, an amiloride-resistant species, is sensitized to the drug by expression of *bsu1*⁺ (Fig. 5D). This shows that amiloride resistance in *S. cerevisiae* derives from inefficient uptake and confirms that amiloride toxicity is caused by interference with intracellular processes (15).

Our data also disprove the speculation that PN uptake in *S. pombe* is mediated by Na⁺ symport (48). This speculation was based on the fact that amiloride is known to inhibit a large variety of Na⁺-dependent transport processes such as Na⁺ channels, Na⁺/H⁺ and Na⁺/Ca²⁺ antiporters, and the Na⁺/K⁺ ATPase, as well as Na⁺ solute symporters (20). However, we found that PN in *S. pombe* is transported by H⁺ symport (Fig. 3C) and amiloride inhibits PN uptake by a competitive mechanism (Fig. 5B). It has recently been demonstrated that PN uptake in mammalian cells is also catalyzed by proton symport and is sensitive to amiloride (33). Together, these findings may indicate that the PN transporters of mammals and fission yeast are structurally related. Because no mammalian homologue of Bsu1p can be identified in database searches, this similarity may, however, be very limited at the level of the primary structure.

Interactions of thiamine and PN metabolism. An unusual feature of the *bsu1*⁺ gene is that its expression strongly responds to thiamine (Fig. 2 and 4) (29). This regulation may be mediated by a conserved DNA element identified in the promoters of *bsu1*⁺ and other thiamine-regulated genes (50).

bsu1⁺ expression also responds to the availability of PN. The effect of PN-deficient medium on the expression of *bsu1*⁺ can be seen in wild-type cells but is strongly increased when PN biosynthesis is absent because of deletion of *snz1*⁺ (Fig. 4). It was recently discovered that PN is the precursor of the pyrimidine unit of thiamine (49). Thus, PN deficiency also leads to thiamine deficiency and the response of *bsu1*⁺ to the different PN concentrations may reflect the changes in the availability of thiamine caused by the different growth regimens. Another consequence of this interrelationship is that thiamine biosynthesis, which is required in the absence of an extracellular supply of thiamine, curtails the concentration of PN present in the cell. In *S. pombe*, this is compensated for by increasing the expression of *bsu1*⁺, allowing a higher rate of PN import. To a smaller extent, the expression of *snz1*⁺ is also increased in thiamine-free medium (data not shown). *S. cerevisiae* cells, in contrast, use a different strategy to acquire more PN when thiamine becomes scarce. Whereas *SNO1* and *SNZ1* are always expressed, the *SNZ2-SNO2* and *SNZ3-SNO3* genes are not expressed in medium containing sufficient thiamine but are highly expressed in the absence of thiamine (32). We could not find any indication that expression of the *S. cerevisiae* PN transporter gene *TPN1* is under the control of thiamine (T. Schwarzmüller and J. Stolz, unpublished results). Thus, in *S. cerevisiae*, PN for thiamine biosynthesis is derived from PN biosynthesis, whereas it is largely derived from PN uptake in *S. pombe*.

It has been noted before that PN and thiamine show a complex interaction with respect to the growth of yeast cells. In some species of the genus *Saccharomyces*, the presence of thiamine in the absence of PN inhibits growth (34). It can be speculated that the species that show this behavior possess only thiamine-repressible alleles of the *SNZ* or *SNO* gene and run out of PN when thiamine is added. For other species, however, thiamine and PN were found to be equivalent and able to replace each other (25). This is likely explained by a sparing effect of thiamine on PN biosynthesis or may even mean that these species, unlike *S. pombe*, have a pathway to produce PN from thiamine. It is not known if this may occur by a reversal of the thiamine biosynthetic pathway or if additional proteins are necessary. In summary, our results show that *S. cerevisiae* and *S. pombe* possess different proteins that act as PN transporters and that the two yeasts also use different strategies to maintain the cellular pool of PN when thiamine biosynthesis is required.

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