

Saccharomyces cerevisiae YOR071C Encodes the High Affinity Nicotinamide Riboside Transporter Nrt1^{*[S]}

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NAD⁺ is an essential coenzyme for hydride transfer enzymes and a substrate of sirtuins and other NAD⁺-consuming enzymes. Nicotinamide riboside is a recently discovered eukaryotic NAD⁺ precursor converted to NAD⁺ via the nicotinamide riboside kinase pathway and by nucleosidase activity and nicotinamide salvage. Nicotinamide riboside supplementation of yeast extends replicative life span on high glucose medium. The molecular basis for nicotinamide riboside uptake was unknown in any eukaryote. Here, we show that deletion of a single gene, YOR071C, abrogates nicotinamide riboside uptake without altering nicotinic acid or nicotinamide import. The gene, which is negatively regulated by Sum1, Hst1, and Rfm1, fully restores nicotinamide riboside import and utilization when resupplied to mutant yeast cells. The encoded polypeptide, Nrt1, is a predicted deca-spanning membrane protein related to the thiamine transporter, which functions as a pH-dependent facilitator with a K_{m} for nicotinamide riboside of 22 μ M. Nrt1-related molecules are conserved in particular fungi, suggesting a similar basis for nicotinamide riboside uptake.

NAD⁺ and the phosphorylated and reduced derivatives, NADP⁺, NADH, and NADPH, are essential coenzymes for hydride transfer enzymes and essential substrates of NAD⁺-consuming enzymes, including sirtuins, ADP-ribose transferases, poly(ADP-ribose) polymerases, and cyclic ADP-ribose synthases (1). In most fungi and vertebrates, *de novo* NAD⁺ synthesis is derived from tryptophan utilization. *De novo* synthesis is sufficient for viability and supplies a yeast cell with ~0.8 mM intracellular NAD⁺. However, supplementation of yeast with nicotinamide riboside (NR),⁴ a newly identified eukaryotic NAD⁺ precursor

(2), increases intracellular NAD⁺ levels and Sir2 activity and extends replicative life span (3).

Nicotinic acid (NA) was discovered as a vitamin in 1938 (4), and the enzymology of NA utilization was sketched out in 1958 as a pathway common to yeast and vertebrates (5). Nicotinamide (Nam), also a classically identified NAD⁺ precursor vitamin (4), is utilized in yeast via NA salvage after nicotinamidase activity (6–8). Nam is used in vertebrate cells via conversion to NMN (9). NR utilization by yeast cells, first demonstrated in 2004, depends on eukaryotic NR kinase Nrk1 or Nrk2 (2). A second pathway for NR utilization is initiated by Urh1 and Pnp1, which split NR into Nam, followed by Nam salvage (3, 10, 11). NA riboside is yet another salvageable precursor converted to NAD⁺ in pathways initiated by Nrk1, Urh1, and Pnp1 (12).

In yeast, submicromolar NA is imported by the high affinity Tna1 permease, which is transcriptionally up-regulated by low NA (13). The molecular basis for import of Nam, NR, and NA riboside is not known in any eukaryotic system. Although NR is found in milk (2), can protect transected dorsal root ganglion neurons from degeneration (14), and extends yeast life span on high glucose medium (3), it is not known how widespread the compound is in nature or whether there is a specific transport system. Here, we discovered that Nrt1, a predicted deca-spanning membrane protein encoded by the YOR071C gene and described previously as low affinity thiamine transporter Thi71 (15), is responsible for high affinity NR uptake and is both necessary and sufficient for NR utilization. Gene expression of Nrt1 and the acid-dependent nature of NR import establish the specificity and regulation of the first step in NR utilization in the yeast system.

EXPERIMENTAL PROCEDURES

Saccharomyces cerevisiae Strains, Plasmids, and Medium—Yeast strains were derivatives of the laboratory wild-type strain BY4742. Single deletion strains have been described (16), and additional strains were generated by one-step gene disruption (17). Plasmid pNRT1, carrying NRT1 under the control of its own promoter, was created by amplifying the gene from BY4742 DNA using primers 14112 and 14113. After digestion with SacI and HindIII, the product was inserted into pRS317. NA-free synthetic glucose complete medium (3) and NR (2) were prepared as described. [³H]NMN was purchased from Moravak Biochemicals (Brea, CA). Growth curves were measured from overnight cultures diluted to $A_{600\text{ nm}} = 0.2$. A yeast strain list and primer sequences are provided in the supplemental table.

Intracellular NAD⁺ and NR Transport—Intracellular NAD⁺ was calculated for cells grown to $A_{600\text{ nm}} = 1.0$ as described (3). For measurement of NR uptake, cells were grown in NA-free medium with rigorous aeration to $A_{600\text{ nm}} = 1.0$, at which time 1 ml of cell culture was combined with the appropriate amount of [³H]NR and incubated for the specified times. Triplicate cell samples were collected using a Millipore 1225 sampling vac-

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[S] The on-line version of this article (available at <http://www.jbc.org>) contains a supplemental table.

This paper is dedicated to the memory of Professor Arthur Kornberg, pioneer in NAD⁺ metabolism.

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⁴ The abbreviations used are: NR, nicotinamide riboside; NA, nicotinic acid; Nam, nicotinamide.

uum manifold on premoistened 1.2- μm nitrocellulose filters and washed twice with 5 ml of potassium-buffered saline prior to scintillation counting. Kinetic parameters were calculated from initial rates. For pH-dependent transport assays, the culture medium was buffered to the indicated pH values by addition of a 1% volume of sodium citrate (pH 3.5–5.5) or Tris-Cl (pH 6.5–9.5).

RESULTS AND DISCUSSION

Nrt1 Is Necessary and Sufficient for Normal NR Incorporation into NAD⁺—The NR transporter in *Hemophilus influenzae* is encoded by the *pnuC* gene (18), which has homologs in eubacteria and bacteriophage. There are no reports of a eukaryotic NR transporter and no *pnuC*-homologous eukaryotic sequences. Overexpression studies of human nucleoside transporters in frog oocytes indicated that high expression of the equilibrative transporters ENT1 and ENT2 and the concentrative transporter CNT3 increased the import of the NR analogs tiazofurin and benzamide riboside (19). Human ENT1 and ENT2 have sequence similarity to Fun26, a broad specificity nucleoside transporter expressed predominantly in intracellular membranes (20). On the basis of the dearth of homology-based candidates for a yeast NR transporter, we assembled a set of 14 single mutants in putative transporter genes, including *fun26* and *fui1*, a reported uridine transporter (20); *tna1*, the NA transporter (13); *dal4*, a reported allantoin/uracil permease (21); *dal5*, a reported ureidosuccinate/allantoate permease (22); *fcy2*, *fcy21*, and *fcy22*, reported purine/cytosine permeases (23); *fur4*, a uracil permease (24); *thi7*, *YOR071C*, and *thi72*, the thiamine transporter and related molecules (15, 25); and *thi73* and *yil166c*, which resemble additional uncharacterized transporters.

Wild-type yeast cells grown on NA-free medium have an intracellular NAD⁺ concentration of ~ 0.8 mM, which is elevated by ~ 1 mM when supplemented with $10 \mu\text{M}$ NR (3). Yeast strains deleted for each of the 14 candidate transporters were assayed for diminution or loss of this effect. As shown in Fig. 1A, all but two candidates were as sensitive to NR as the wild type. Deletions in *nrt1* (previously termed *YOR071C/THI71*) and *fun26* decreased incorporation of NR into NAD⁺ by 83 and 36%, respectively. As shown in Fig. 1B, the double *nrt1 fun26* mutant exhibited a 93% reduction in NR utilization, rendering the NR-dependent increase in NAD⁺ concentration statistically insignificant. These data suggest that Nrt1 is responsible for the majority of NR import, with a potential minor role for Fun26.

To test the hypothesis that NR utilization depends on Nrt1, the gene was cloned into the single copy vector pRS317 under the control of its native promoter. As shown in Fig. 1B, upon introduction of this plasmid into the double mutant background, there was a 102% restoration of the NR-dependent increase in NAD⁺.

To test whether Nrt1 and Fun26 might have roles in assimilation of other NAD⁺ precursors, we grew wild-type and *nrt1 fun26* mutant strains in NA-free medium and in medium supplemented with $10 \mu\text{M}$ NR, NA, or Nam. Whereas the wild-type strain incorporated each vitamin into an additional 1 mM intracellular NAD⁺, the *nrt1 fun26* strain obtained the full NAD⁺

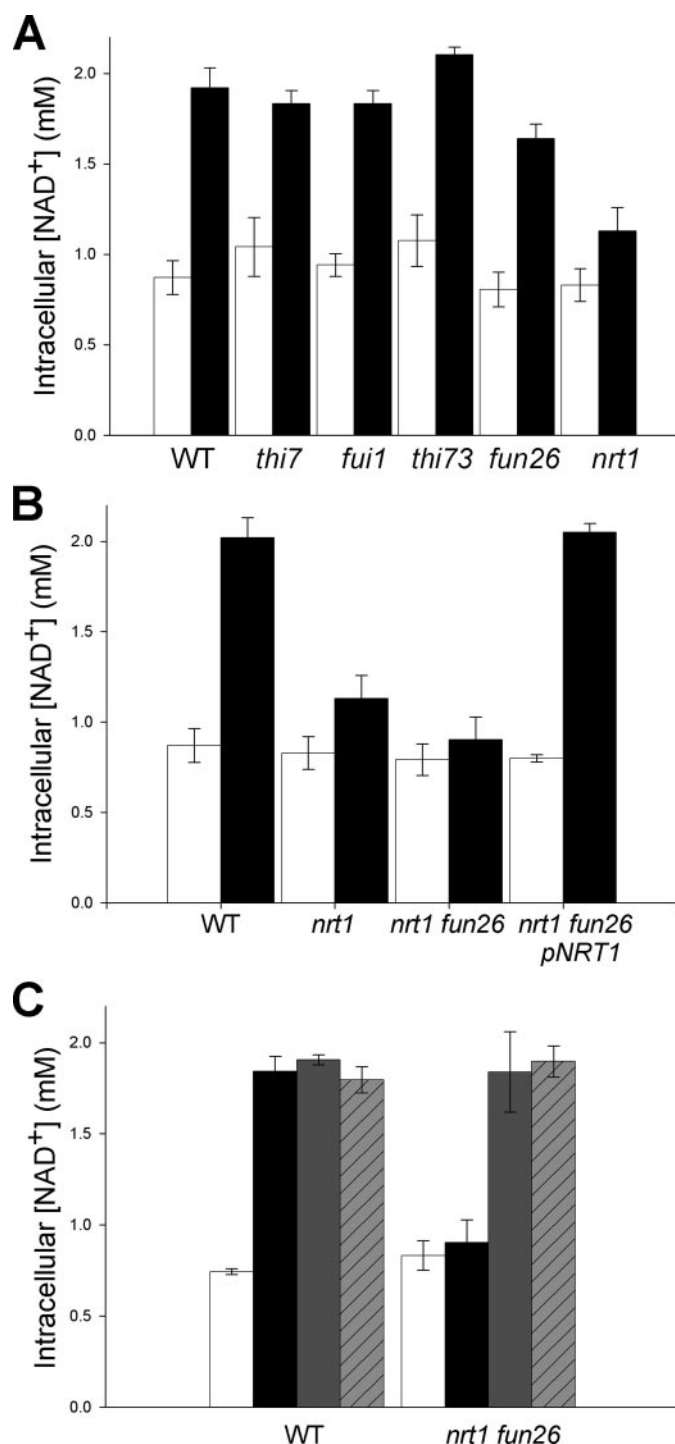


FIGURE 1. Nrt1 is specifically required for NR utilization. A, the intracellular NAD⁺ concentration was determined in yeast strains of varying genotype in NA-free medium (white bars) and in medium supplemented with $10 \mu\text{M}$ NR (black bars), demonstrating a minor defect in NR utilization in the *fun26* mutant and a major defect in NR utilization in the *nrt1* mutant. B, wild-type (WT) NR utilization was restored in the *nrt1 fun26* mutant by cloned *NRT1*. C, whereas a wild-type strain could elevate intracellular NAD⁺ by 1 mM with addition of $10 \mu\text{M}$ NR (black bars), $10 \mu\text{M}$ NA (gray bars), or $10 \mu\text{M}$ Nam (hatched bars) to NA-free medium (white bars), the *nrt1 fun26* mutant was specifically deficient in NR utilization and had no defect in utilization of NA or Nam.

benefit from NA and Nam, but not from NR (Fig. 1C). Thus, neither Nrt1 nor Fun26 appears to play any role in import of NA or Nam.

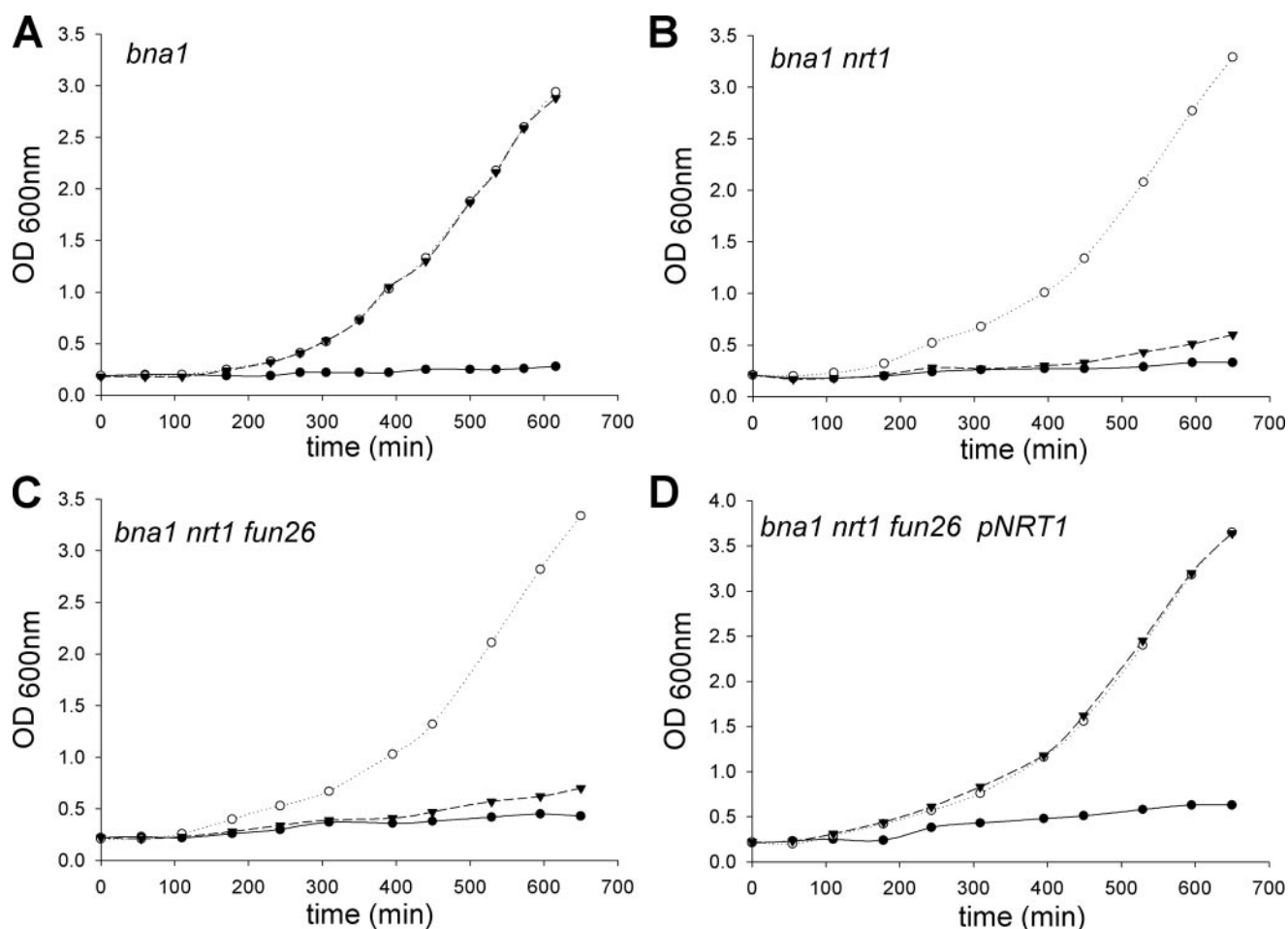


FIGURE 2. **Nrt1 is required for growth on NR.** A, growth of mutants in *de novo* NAD⁺ biosynthesis depended on addition of 10 μ M NR (▼) or 10 μ M NA (○) with respect to NA-free medium (●). Deletion of *nrt1* (B) or *nrt1* and *fun26* (C) rendered the *bna1* mutant NA-dependent and NR-nonresponsive for growth. D, addition of plasmid-borne *NRT1* restored NR-responsive growth to *bna1 nrt1 fun26*.

Nrt1 Is Necessary and Sufficient for NR-dependent Cell Growth—To set up a system in which deletions of putative vitamin transporter genes could be assayed for effects on cell growth, we deleted the gene encoding 3-hydroxyanthranilic acid dioxygenase, which performs an essential step in the *de novo* biosynthesis of NAD⁺ from tryptophan. As shown in Fig. 2A, the *bna1* mutant was incapable of growth in NA-free medium but grew well when supplemented with either 10 μ M NA or NR. In contrast, as shown in Fig. 2 (B and C), deletion of the *NRT1* gene with or without deletion of *FUN26* permitted NA-dependent growth but abolished normal growth with NR supplementation. When the *NRT1* gene was added back to the *bna1 nrt1 fun26* mutant (Fig. 2D), NR-dependent growth was fully restored. Indeed, the Nrt1 dependence of NR utilization is stronger than the Tna1 dependence of NA utilization because *bna1 tna1* mutants fail to grow at 40 nM NA, grow slightly at 400 nM NA, and grow normally at 4 μ M NA (13). In contrast, examination of the growth curves in Fig. 2 (B and C) indicates that growth of *bna1 nrt1* mutants was slight at 10 μ M NR.

Nrt1 Is Required for High Affinity, pH-dependent, Specific Import of NR—To determine whether NR is incorporated into yeast cells in an Nrt1-dependent manner, we prepared [³H]NR from [³H]NMN and measured incorporation of radioactivity

into yeast cells exposed to 50 μ M NR. The wild-type strain exhibited a robust linear import activity for the entirety of the 70-min assay, whereas the *nrt1* disruption strain exhibited no detectable import (Fig. 3A).

Nrt1 is highly similar in sequence to Thi7, a concentrative thiamine transporter and member of the major facilitator superfamily (15) with an ability to concentrate thiamine 1000-fold inside the cell (26). Thiamine import is pH-dependent, with strong activity between pH 4 and 5 and declining activity at pH 6 and 7 (26). Additionally, Nrt1 is related in sequence to Fur4, which transports uracil via a proton symport mechanism (27). Accordingly, we tested the hypothesis that Nrt1 transport of NR is pH-dependent. Although initial rates of 25 μ M NR import were essentially unchanged from pH 3.5 to 6.5, the import of NR was reduced to 5% at pH 7.5 and not distinguishable from the background at pH 8 and above (Fig. 3B).

Having shown that all detectable NR import is Nrt1-dependent, we were able to characterize the kinetic parameters of NR transport by Nrt1 in wild-type cells over a range of NR concentrations. Transport was saturable with a K_m of 21 ± 3.6 μ M and a maximal rate of 20.4 ± 0.9 pmol/min/ 10^7 cells (Fig. 3C). Taking the intracellular volume of a haploid cell to be 70 fl (28), the rate of maximal import would produce ~ 29 μ M NR inside the

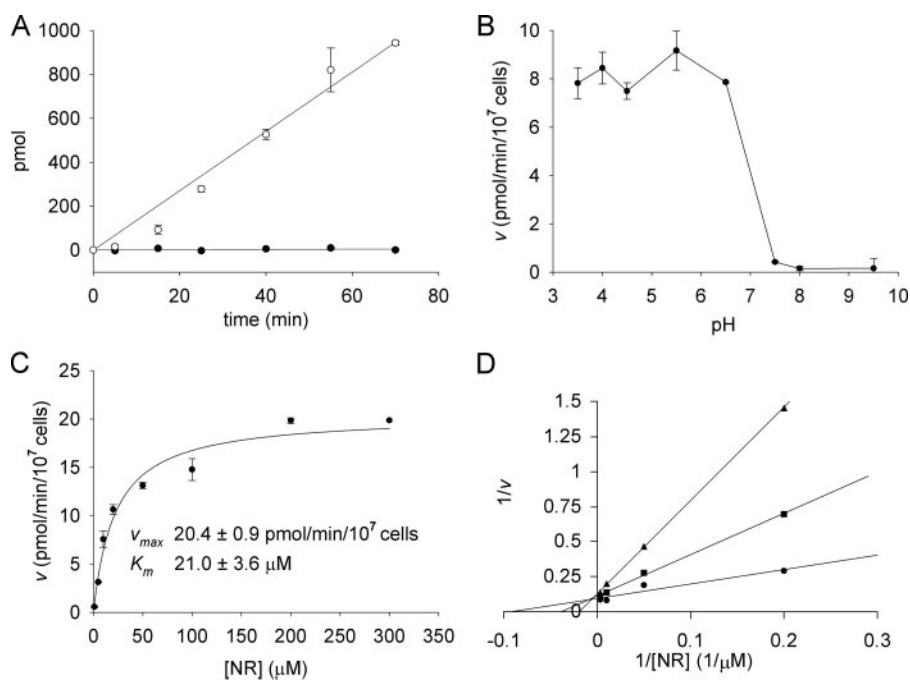


FIGURE 3. Kinetics of Nrt1, the high affinity NR transporter. *A*, time course of [^3H]NR uptake by wild-type (\circ) and *nrt1* (\bullet) cells. *B*, pH dependence of [^3H]NR uptake by wild-type cells. *C*, concentration dependence of NR uptake kinetics by wild-type cells. *D*, Lineweaver-Burk plot of NR uptake as a function of NR concentration (\bullet). Experiments performed in the presence of 10 mM Nam (\blacksquare) and 10 mM NMN (\blacktriangle) indicate purely competitive inhibition (identical y intercept) with K_i values >2 mM.

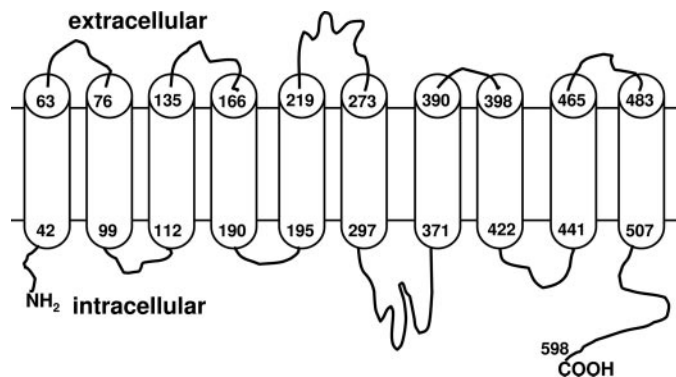


FIGURE 4. Predicted 10-transmembrane topology of Nrt1. The predicted topology of the Nrt1 protein from hidden Markov modeling (31) is depicted schematically.

cell/min. Under standard supplementation conditions of $10\ \mu\text{M}$ extracellular NR, $\sim 9\ \mu\text{M}$ nucleoside would be imported per min.

In addition to the pH dependence of NR transport, we considered that NR transport may be under the regulation of a set of transcriptional repressors that control expression of multiple components of the NAD^+ biosynthetic machinery. Examination of the microarray data from cells deleted for either the transcriptional repressor Sum1, the NAD^+ -dependent protein lysine deacetylase Hst1, and Rfm1, which forms a protein complex with Sum1 and Hst1, indicates that *NRT1* is one of 55 genes derepressed by deletion of each factor (29). These data suggest that NR transport capacity may be derepressed at the transcriptional level under conditions of declining NAD^+ , when Hst1 enzyme activity becomes limited (30).

Once NR enters the cell, it is converted to NMN (2) or Nam (3). To test whether metabolites related to NR inhibit NR transport, non-labeled Nam and NMN were added to NR import assays. At high micromolar concentrations, no inhibition was detected. However, at Nam and NMN concentrations of 10 mM, competitive inhibition was demonstrated (Fig. 3D), albeit with K_i values >2 mM.

Sequence Analysis of Nrt1—By hidden Markov modeling and topology prediction (31), Nrt1 is a deca-spanning membrane protein with both termini and a 74-amino acid loop between helices 6 and 7 projecting into the cytoplasm (Fig. 4). This topology is the same as the 28% sequence identical Fur4 uracil-proton symporter, the internalization of which is mediated by cytoplasmic phosphorylation and ubiquitylation (32). The closest sequence homologs of Nrt1 are Thi7 (84%), the thiamine transporter, and Thi72 (81%), which, like Nrt1, has been considered a low affinity thiamine transporter (15). These data suggest recent gene duplication events and, considering the absence of NR transport in *nrt1* mutants, functional divergence. In the pathogenic yeast *Candida glabrata*, NR availability appears to play a critical role in host infection (11). Thus, it is interesting to note that the sequences most similar to Nrt1 outside of *S. cerevisiae* Thi7 and Thi72 are *C. glabrata* sequences XP_446731 (68%) and XP_449349.1 (65%) and the *Vanderwaltozyma polyspora* sequence XP_001645454.1 (67%).

Identification of Nrt1 as the high affinity NR transporter in *S. cerevisiae* reinforces the need to identify environmental sources of NR. Current data indicate that NR utilization is limited by alkaline conditions and by Nrt1 expression, which is repressed by Sum1, Hst1, and Rfm1 (29). Additional work will be needed to define the effect of cell aging and nutritional conditions on NR transport, the influence of cellular metabolism on Nrt1 internalization, and the identity of NR transporters in other organisms.

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