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TAT1 Encodes a Low-Affinity Histidine Transporter in *Saccharomyces cerevisiae*

Milan Bajmoczi, Mary Sneve, David J. Eide,[1] and Lester R. Drewes[2]
Department of Biochemistry & Molecular Biology, School of Medicine, University of Minnesota–Duluth, 10 University Drive, Duluth, Minnesota 55812

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Previous studies have revealed the presence of at least two histidine uptake systems in *S. cerevisiae*; one with high affinity and the other with low affinity for histidine. The *HIP1* gene is known to encode the high affinity permease. The purpose of this study was to identify the gene that encodes the low affinity permease. A mutant strain of *S. cerevisiae* that is both a histidine auxotroph and a *hip1* deletion mutant is unable to grow on low histidine media. This strain was transformed with a yeast cDNA library constructed in a yeast expression vector. Transformants with increased histidine transport were selected by their ability to grow on a low histidine media. Sequencing of the inserts revealed the presence of the *HIP1* gene and also the presence of the *TAT1* gene. Estimated K_M and V_{max} *values for histidine transport by each system were determined. In a hip1 tat1* double mutant, the level of histidine required for growth increased eight-fold in comparison to the *hip1* single mutant. Our results suggest that the *TAT1*-encoded protein, previously characterized as the high-affinity tyrosine permease, also acts as the low affinity histidine permease.

Amino acids, the building blocks of proteins, are of crucial importance to living organisms. The transport of these entities across the plasma membrane is mediated by proteins, referred to as transporters or permeases, embedded in this membrane. The yeast genome sequencing project resulted in the identification of 6293 open reading frames in the yeast chromosomal genome (1). Extensive database searches as well as binary comparison identified approximately 100 *S. cerevisiae* transport proteins. These were subsequently assigned to 17 families on the basis of amino acid homology and similarities in transported substrates (2). The amino acid permease family consists of several clusters of proteins, with the members of each cluster presumably exhibiting similar transport properties. This project was focused on members of Cluster 1 (2) including the HIP1- high affinity histidine permease (3), the GAP1- general amino acid permease gene (4), the BAP2 - leucine, isoleucine and valine permease (5) and the TAT1 - the high affinity tyrosine permease (6).

Previous studies have shown that the uptake of histidine is mediated by the GAP1 protein and two histidine-specific transport systems (7). The high affinity system was previously described and the gene encoding this protein was named *HIP1* (3). The low affinity permease was competitively inhibited only by tyrosine, suggesting that the tyrosine permease could be the low affinity transporter. The purpose of this project was to identify the gene(s) encoding the permease(s) responsible for uptake of histidine with low affinity. Using the functional complementation technique, two genes whose overexpression complement a *hip1* histidine transport mutant, were isolated. Sequence analysis identified one of the genes as *HIP1*, the known high-affinity permease. The second gene was *TAT1*, the gene previously determined to encode the high affinity tyrosine permease (6). Our results suggest that the *TAT1* gene also encodes the with low affinity histidine transporter.

MATERIALS AND METHODS

Strains, cDNA library, and culture methods. The JT16 yeast strain used in this study [*MAT* **a** *hip1-614 his4-401 ura3-52 ino1 can1*] (3) was provided by G. Fink (Whitehead Institute, Cambridge, MA). A *Saccharomyces cerevisiae* cDNA library (8) constructed in the pRS316-GAL1 vector was obtained from A. Bretscher, Cornell University, Ithaca, NY. Insert expression was regulated by the galactose-inducible *GAL1* promoter (8). The vector has *URA3* and Amp markers, which enable the plasmid to complement the *ura3* pheno-

[1] Current address: Nutritional Sciences Program, 217 Gwynn Hall, University of Missouri–Columbia, Columbia, MO 65211.
[2] Corresponding author. Fax: (218) 726-8014. E-mail: ldrewes@d.umn.edu.

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type in yeast and to confer ampicillin resistance in *E. coli*. JT16 was grown on 1 % yeast extract, 2 % peptone, 2 % glucose medium supplemented with 650 μM histidine (3). The transformants were selected on SC ura$^-$ medium consisting of 90 mg/ L each: adenine, arginine, aspartic acid, histidine, inositol, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, valine, 0.67% yeast nitrogen base without amino acids and with ammonium sulfate and either 4 % galactose or 2 % glucose. Ammonium sulfate was added to suppress both the expression and the uptake activity of the GAP1 permease (9), which represents an alternative pathway for histidine uptake. The Ura+ transformants were screened on SC ura$^-$+ 600 μM histidine for clones that would also complement the *hip1* phenotype. Plasmids were selectively removed from JT16 using 5-fluoroorotic acid (10). Cell number in liquid cultures was determined by measuring the optical density of cell suspensions at 600 nm (A_{600}). A standard curve was used to convert these values into cell numbers. Plasmids were propagated in *Escherichia coli* TOP10F' (Invitrogen Corp.) on Luria-Bertani medium supplemented with ampicillin (50 μg/ ml).

Transformation and screening. The *S. cerevisiae* library was screened for genes encoding proteins that functionally complemented the *hip1* phenotype. The level of histidine at which the growth of the recipient strain JT16 is limited was determined by performing a titration with histidine levels ranging from 15 mM to 0.02 mM. Transformation, using the lithium acetate method (11), was used to introduce the cDNA library into JT16 cells. Transformants selected on SC ura$^-$medium were washed with 2 ml of sterile water and plated on SC ura$^-$600 μM histidine medium. DNA from the colonies growing on the latter media, was prepared and transformed into *E. coli* (12). Restriction analysis using two pairs of enzymes, *Bam*HI/*Not*I and *Bgl*II/*Nco*I, was used to identify related clones. Three representatives of each group obtained were sequenced on an ABI 377 Automated DNA Sequencing Analyzer (Advanced Genetic Analysis Center, St. Paul, MN).

Uptake assay. Cells were grown to saturation in 5 ml of SC ura$^-$600 μM histidine liquid medium. The candidates were then inoculated into 50 ml of the same medium starting at A_{600} = 0.05. (pRS316-GAL1 vector transformed control cells were grown in the medium supplemented with 15 mM histidine.) The cells were harvested at culture A_{600} values between 1 and 3, washed three times in 0.67 % yeast nitrogen base without amino acids (YNB) and resuspended at A_{600} = 80. The reaction was initiated by diluting 30 μl of prepared cells into 120 μl of YNB containing 0.5 μCi ^{3}H labeled histidine and a sufficient amount of cold histidine to obtain the desired final concentration. For the time-dependent uptake studies, the concentration was kept constant at 600 μM histidine and the duration of the assay was varied. For the concentration-dependent studies, the time of the incubation was kept constant at 6 minutes and only the concentration of histidine was varied. The reaction was terminated at the desired time by adding 4 ml of ice cold buffer to the assay mixture. Cells were recovered by vacuum filtration (Whatman, 2.4 cm glass fibre paper), washed with 5 ml of ice-cold buffer, transferred into 5 ml of scintillation fluid, and after 30 hours the accumulated radioactivity was measured by scintillation counting. The experiments were performed with triplicate samples.

Construction of hip1Δ tat1::URA3 double mutant (MB1). A plasmid containing a *tat1::URA3* disruption mutation was kindly provided by A. Schmidt, University of Basel, Basel, Switzerland. A DNA fragment containing the *tat1::URA3* allele was obtained by cutting this plasmid with *Eco*RI and the fragment was purified following gel electrophoresis using the Wizard PCR Preps kit (Promega). One μg of the *tat1::URA3* fragment was transformed into JT16 and plated onto SC ura$^-$ medium + 15 mM histidine. Three *ura+* colonies were picked and their genomic DNA was isolated. Polymerase chain reaction (PCR) was used to verify the incorporation of *TAT1::URA3* allele into the genome of the candidates in exchange for the wild type *TAT1* gene. The primers used in this procedure were complementary to the *TAT1* gene and separated by approximately 800 b.p. in the wild type gene and by approximately 2000 b.p. in the *tat1::URA3* mutant. Two candidates, determined to be positive transformants by PCR, were then plated on SC ura$^-$medium with ammonium sulfate and with the level of histidine varying from 0 to 15 mM.

RESULTS AND DISCUSSION

Isolation of the TAT1 gene. JT16, a yeast strain lacking both the high affinity histidine permease and the histidine biosynthetic pathway represented an ideal strain for identifying the low affinity permease by complementation. The strategy was to screen the *Saccharomyces cerevisiae* library for genes encoding proteins that would functionally complement the *hip1* phenotype. The first step was to determine the level of histidine at and below which the growth of the recipient strain JT16 is limited. The titration performed with the levels of histidine varying from 15 mM to 0.02 mM indicated that 600 μM was the limiting level. Plating of transformed JT16 cells onto SC ura$^-$medium yielded approximately 10^6 Ura+ transformants per 1 μg of cDNA library. The cells were collected and spread onto 5 SC ura$^-$+ 600μM histidine plates at about 2×10^5 cells/plate. Thirty-six colonies were identified on this medium. To verify that the growth of these candidates

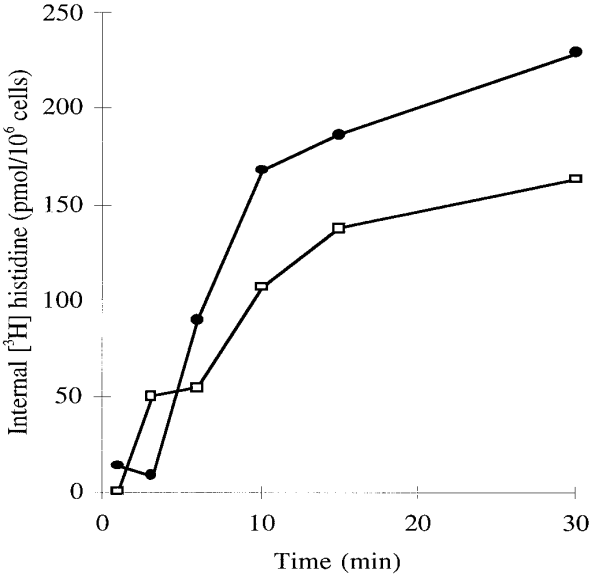

FIG. 1. Time dependence of histidine uptake by cells overexpressing either *HIP1* (closed circles) or *TAT1* (open squares). Histidine accumulation by the control cells at the corresponding concentrations were subtracted from the values obtained from the overexpressing cells to yield the adjusted values shown. The amount of histidine accumulated by control cells JT16 was on average 18 % of the amount accumulated by *TAT1*-overexpressing cells and 6 % of the amount accumulated by the *HIP1*-overexpressing cells. Each point represents the average of three measurements.

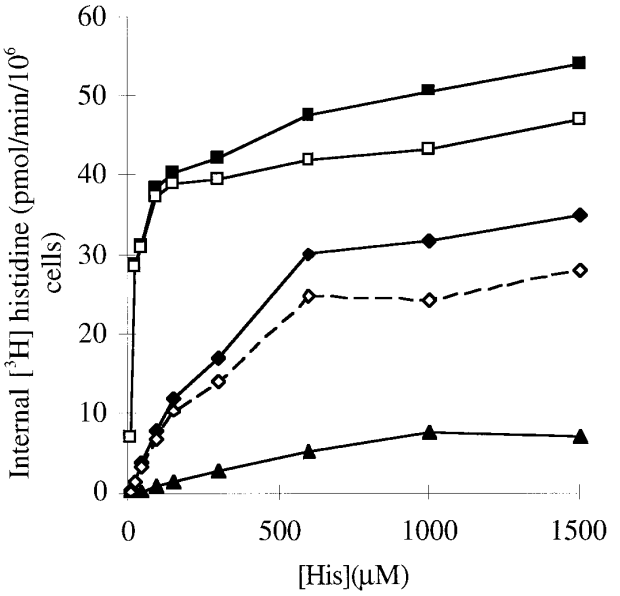

FIG. 2. Michaelis-Menten concentration dependence plot of histidine uptake by cells overexpressing either *HIP1* or *TAT1*. The histidine accumulations by the control cells JT16 (closed triangles) at the corresponding concentrations were subtracted from the values obtained originally by the *HIP1* or *TAT1*-overexpressing cells (solid lines with closed squares and closed diamonds respectively) to yield the adjusted values shown. The dashed lines represent the accumulation of histidine in the *HIP1*-overexpressing cells (open squares) and the *TAT1*-overexpressing cells (open diamonds) following that adjustment. Each point represents the average of three measurements.

required expression of the plasmids cDNA inserts, the colonies were plated on SC ura$^-$+ 600μM histidine medium with 2% glucose. The GAL1 promoter is repressed in the presence of glucose and the expression of the inserts is low (3). Eleven candidates that grew equally well or better on glucose medium, in comparison to galactose medium, were not analyzed further. When the plasmids were selectively removed from the remaining 25 strains with 5-fluoroorotic acid (5-FOA), three candidates retained their ability to grow on 600 μM histidine medium. Analysis of these three plasmid-independent clones was not continued. Because JT16 is both a histidine auxotroph and an uptake defective mutant (1), it is possible that positive transformants complemented the biosynthetic pathway defect rather than histidine transport. Nine of the remaining candidates exhibited growth on a histidine-free SC ura$^-$ medium and were assumed to have the *HIS4* insert. For the remaining 13 candidates, plasmid DNA was extracted and then reintroduced into JT16. All retransformants exhibited the galactose-dependent suppression of *hip1* phenotype. Restriction analysis was used to evaluate similarities among the remaining candidates. This procedure showed that the remaining candidates all fell into one of two groups. The inserts of three candidates from each group were partially sequenced. Comparison of the obtained sequences with the known open reading frames of the yeast genome in the GenBank revealed the presence of *HIP1* insert in one of the groups and *TAT1* in the second.

Uptake studies. Our uptake studies indicated that the product of the *TAT1* gene is directly involved in the low affinity uptake of histidine. Histidine uptake was assayed to determine the effect of *HIP1* or *TAT1* overexpression on this process. The amounts of histidine accumulated internally in control cells, represented by JT16 transformed with pRS316-GAL1 without an insert, were subtracted from the values obtained with JT16 overexpressing a wild type *HIP1* or *TAT1* gene. In this way, the data represent histidine uptake activity due only to the overexpressed gene product. The time-dependent studies reveal that at saturation, more histidine is accumulated in the cells that have overexpressed either *HIP1* or *TAT1* gene (150 pmol/10^6 cells and 250 pmol/10^6 cells respectively) relative to control values (Fig.1). The time-dependent uptake studies also show that HIP1-dependent uptake reaches a plateau at approximately 10 minutes after addition of cells. Approximately 15 minutes is needed for the overexpressed *TAT1* gene product to reach the saturation level. The Michaelis-Menten plot (Fig.2) provides further evidence that the uptake of histidine is enhanced by both *HIP1* and *TAT1* gene products. When assayed at 600 μM histidine, the cells bearing the plasmid with *TAT1* insert accumulated histidine at a rate,

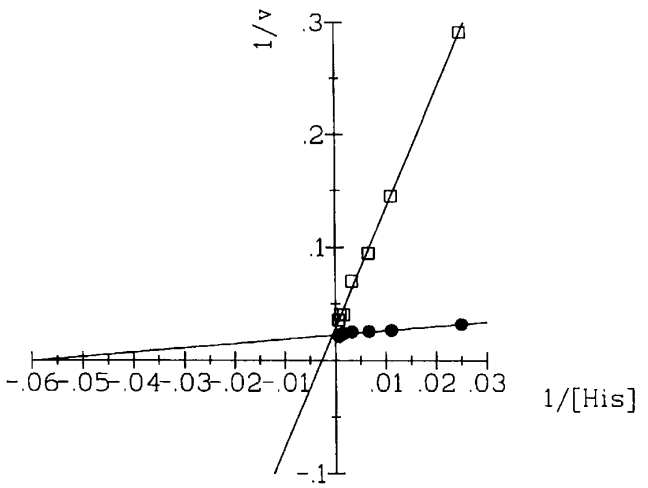

FIG. 3. Kinetic analysis of histidine uptake in cells overexpressing either *HIP1* or *TAT1*. The adjusted data from the Michaelis-Menten concentration dependence plot in Fig. 2., in which *HIP1* and *TAT1* transformants were assayed for histidine uptake rates over a range of histidine concentrations, were used to generate the Lineweaver-Burke reciprocal plot shown, in which the *HIP1* and the *TAT1*-overexpressing cells are represented by closed circles and open squares respectively. ([His] in μM, v in pmol/min/10^6 cells.)

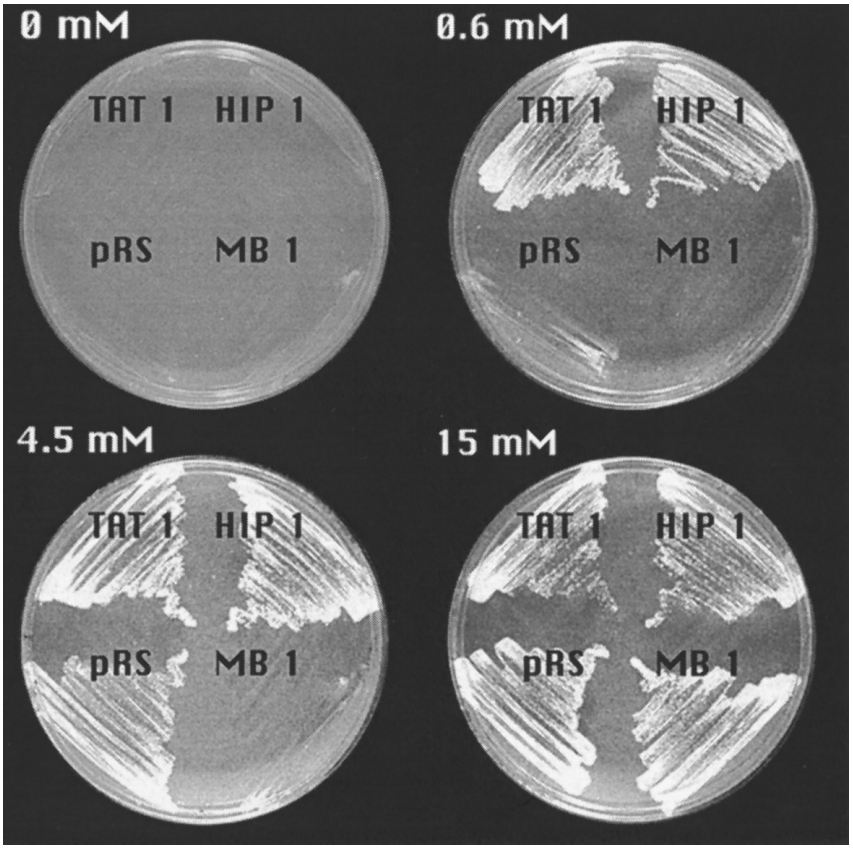

FIG. 4. Growth characteristics on different levels of histidine. The *TAT1*-overexpressing JT16 cells (TAT1), the *HIP1*-overexpressing JT16 cells (HIP1), the pRS316-GAL1 vector JT16 transformants (pRS) and MB1 cells (MB1) were plated on various levels of histidine. The histidine levels are indicated in the upper left corner to the corresponding plates.

approximately 25 pmol/min/10^6 cells, greater than JT16. A difference of 42 pmol/min/10^6 cells is seen for the cells overexpressing *HIP1*. Presumably, these differences are of a sufficient magnitude to allow the cells overexpressing *HIP1* or *TAT1* to grow on the level of histidine at which the control cells JT16 are unable to grow. The Lineweaver-Burke plot (Fig.3) was employed to estimate the apparent K_M and V_{max} values. The estimated K_m values for HIP1 and TAT1 are 17 μM and 370 μM respectively. The estimated V_{max} values are 44 pmol/min/10^6 cells for HIP1 dependent uptake and 35 pmol/min/10^6 cells for TAT1 dependent uptake.

MB1 strain. To further assess the role of the *TAT1*-encoded protein as the low affinity permease, a *hip1*Δ *tat1::URA3* double mutant was constructed. Plating of two double mutant isolates on various levels of histidine demonstrated that the *hip1 tat1* strain requires greater than 4.5 mM histidine to grow relative to only 600 μM for the *hip1* single mutant (Fig. 4). This represents an eight-fold increase in histidine requirement and provides further evidence that the *TAT1* gene product is involved in the uptake of histidine in *S. cerevisiae.* However, at the level of histidine equal to 15 mM, even the double mutant strain MB1 grows, indicating the existence of at least one additional route for histidine entry.

The complementation approach used in these studies revealed that overexpression of either *HIP1* or *TAT1* allows for cell growth even at the level of histidine at which the control *hip1* cells are unable to live. Furthermore, it was shown that mutating the *TAT1* gene results in an eight-fold elevation of the minimum level of histidine necessary for growth of the *hip1* mutant. This study shows that the high-affinity tyrosine permease, encoded by *TAT1* gene, is also involved in low-affinity histidine transport. Further studies are necessary to explore whether the *TAT1* gene product is the only active participant in the low-affinity histidine transport system or whether other proteins are required for this system to function.

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