MOLECULAR AND CELLULAR BIOLOGY, Aug. 1997, p. 4282–4293 Vol. 17, No. 8
0270-7306/97/$04.00+0

A Family of Ammonium Transporters in *Saccharomyces cerevisiae*†

ANNE-MARIE MARINI, SAÏD SOUSSI-BOUDEKOU, STEPHAN VISSERS, AND BRUNO ANDRE*

Laboratoire de Physiologie Cellulaire et de Génétique des Levures, Université Libre de Bruxelles, B-1050 Brussels, Belgium

Received 24 February 1997/Returned for modification 3 April 1997/Accepted 7 May 1997

Ammonium is a nitrogen source supporting growth of yeast cells at an optimal rate. We recently reported the first characterization of an NH_4^+ transport protein (Mep1p) in *Saccharomyces cerevisiae*. Here we describe the characterization of two additional NH_4^+ transporters, Mep2p and Mep3p, both of which are highly similar to Mep1p. The Mep2 protein displays the highest affinity for NH_4^+ (K_m, 1 to 2 μM), followed closely by Mep1p (K_m, 5 to 10 μM) and finally by Mep3p, whose affinity is much lower (K_m, ~1.4 to 2.1 mM). A strain lacking all three *MEP* genes cannot grow on media containing less than 5 mM NH_4^+ as the sole nitrogen source, while the presence of individual NH_4^+ transporters enables growth on these media. Yet, the three Mep proteins are not essential for growth on NH_4^+ at high concentrations (>20 mM). Feeding experiments further indicate that the Mep transporters are also required to retain NH_4^+ inside cells during growth on at least some nitrogen sources other than NH_4^+. The *MEP* genes are subject to nitrogen control. In the presence of a good nitrogen source, all three *MEP* genes are repressed. On a poor nitrogen source, *MEP2* expression is much higher than *MEP1* and *MEP3* expression. High-level *MEP2* transcription requires at least one of the two GATA family factors Gln3p and Nil1p, which are involved in transcriptional activation of many other nitrogen-regulated genes. In contrast, expression of either *MEP1* or *MEP3* requires only Gln3p and is unexpectedly down-regulated in a Nil1p-dependent manner. Analysis of databases suggests that families of NH_4^+ transporters exist in other organisms as well.

Genetic studies and systematic genome sequencing have shown that the yeast *Saccharomyces cerevisiae* possesses many transmembrane transport proteins (2). Not only are yeast transport systems diverse, but they are also frequently multiple; i.e., the same substrate may be transported by several systems with different kinetic properties, specificities, and regulation. The first evidence of permease multiplicity in yeast came from genetic studies of amino acid permeases (54). An example is the transport of γ-aminobutyrate (GABA), an amino acid that can serve as the sole nitrogen source. GABA uptake is mediated by the general amino acid permease (Gap1p), the proline permease (Put4p), and an inducible GABA-specific permease (Uga4p) (3, 22). The most demonstrative example of transporter multiplicity is the HXT family of hexose transporters (7), which includes no fewer than 17 highly similar proteins. Among these, at least HXT proteins 1 through 7 function independently as hexose transporters. The expression and activity of these transporters are regulated by the presence and concentration of glucose in the medium (7, 27, 37, 39). Multiple transport systems with different kinetic properties and regulation likely enable cells to adapt to many different growth conditions (20). In addition, members of some transporter families might instead play a regulatory role in transport, as recently illustrated in the case of the Snf3 and Rgt2 proteins of the HXT family (7, 27, 36, 37).

Glutamine, asparagine, and ammonium are good nitrogen sources supporting optimal growth of yeast cells (12, 54). Addition of NH_4^+ to yeast cells grown on a less-favored nitrogen source triggers nitrogen catabolite inactivation and nitrogen catabolite repression of several enzymes and permeases involved in the utilization of secondary nitrogen sources (54). For instance, synthesis of the general amino acid permease (Gap1) is strongly reduced after addition of NH_4^+, while presynthesized Gap1 is inactivated and subsequently degraded; the latter mechanism involves ubiquitin (19, 23). The central role of NH_4^+ in control of nitrogen metabolism prompted us to characterize the proteins involved in its uptake. High-affinity uptake of NH_4^+ has been shown to involve at least two distinct transporters (Mep1p and Mep2p), and evidence for the existence of a third, lower-affinity system has been produced (14). The *MEP1* gene, encoding a specific, high-capacity transporter with an affinity in the 5 to 10 μM range, has been characterized at the molecular level (34). In a parallel study, the first plant NH_4^+ transporter (Amt1) was cloned by expression in yeast and proved to be very similar in sequence to Mep1p (35). Among several bacterial homologs found by database screening, the Amt1 protein of *Corynebacterium glutamicum* was subsequently shown to display the properties of a (methyl)ammonium transporter (47).

Here we report the molecular, kinetic, and physiological characterization of two additional yeast NH_4^+ transporters, Mep2p and Mep3p, which together with Mep1p constitute a family of three highly similar proteins.

MATERIALS AND METHODS

Strains, growth conditions, and methods. The *S. cerevisiae* strains used in this study are all isogenic with the wild-type strain Σ1278b (5) except for the mutations mentioned (Table 1). Cells were grown in a minimal buffered (pH 6.1) medium with 3% glucose as the carbon source (25). To this medium, one or more of the following nitrogen sources were added: proline (0.1%), glutamate (0.1%), glutamine (0.1%), and $(NH_4)_2SO_4$ (usually the concentration of this salt was 10 mM; occasional modifications of this concentration are mentioned). Yeast cells treated with lithium acetate (24) were transformed as described previously (46). The RNA isolation procedure has also been described previously (46). The

* Corresponding author. Mailing address: Laboratoire de Physiologie Cellulaire et de Génétique des Levures, Université Libre de Bruxelles, Campus Plaine CP 244, Bld. du Triomphe, B-1050 Bruxelles, Belgium. Phone: 32-2-6505411. Fax: 32-2-6505421. E-mail: bran@ulb.ac.be.

† Dedicated to the memory of Marcelle Grenson.

TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference
Σ1278b	*MAT*α	10
23344c	*MAT*α *ura3*	17
21983c	*MAT*α *ura3 gap1-1 can1-1*	17
26972c	*MAT***a** *ura3 mep1-1 mep2-1*	14
AM1	*MAT*α *ura3 mep1*Δ	34
AM2	*MAT***a** *ura3 leu2 mep1*Δ *mep2*Δ*::LEU2*	This study
31000a	*MAT***a** *ura3 leu2 mep1*Δ	This study
31011a	*MAT*α *ura3 mep3*Δ*::KanMX2*	This study
31018b	*MAT*α *ura3 mep2*Δ*::LEU2 mep3*Δ*::KanMX2*	This study
31019b	*MAT***a** *ura3 mep1*Δ *mep2*Δ*::LEU2 mep3*Δ*::KanMX2*	This study
31021c	*MAT*α *ura3 mep1*Δ *mep2*Δ*::LEU2*	This study
31021d	*MAT*α *ura3 mep2*Δ*::LEU2*	This study
31022a	*MAT*α *ura3 mep1*Δ *mep3*Δ*::KanMX2*	This study
SBS21	*MAT*α *ura3 nil1*Δ*::KanMX2*	49
30505b	*MAT*α *ura3 gln3*Δ	49
50027c	*MAT***a** *ura3 leu2 gln3*Δ *nil1*Δ*::KanMX2*	49

Escherichia coli strain used was JM109. All procedures for manipulating DNA were standard ones (4, 44).

Cloning of *mep2*-complementing plasmids. Strain 26972c (*mep1-1 mep2-1 ura3*) was transformed with a low-copy-number plasmid library representing the total genome of wild-type Σ1278b (34). Among the 35,000 Ura^+ transformants, at least 5 displayed growth on two test media: minimal buffered medium with either 1 mM NH_4^+ or 0.1% proline plus 50 mM methylammonium. DNAs isolated from these transformants were used to transform *E. coli*. When reintroduced into strain 26972c, the purified plasmid YCpAM21 carrying a 7-kb genomic insert complemented the defects due to the *mep2-1* mutation. The following oligonucleotide primers were used to amplify the *mep2-1* allele from strain 26972c: 5′-CGCCGGATCCGAATACAGATTGGATGCACTGG-3′ and 5′-ACACAGGCCTGCTTGTCA-3′.

Cloning of the *MEP3* gene. Bacterial clones of a library representing the Σ1278b genome (34) were screened with a γ-^{32}P-radiolabeled oligonucleotide whose sequence was 5′-ACAGATGCACTTCATATACATCAATGC-3′. Three plasmids with partially overlapping probe-positive inserts were isolated. The shortest insert (7 kb, plasmid YCpLEB5) was chosen for further study.

DNA sequencing. Random libraries of plasmids YCpAM21 and YCpLEB5 were prepared as described previously (34). DNA fragments were sequenced by the dideoxy chain termination method (45).

Plasmids. The YCp*MEP2-lacZ* plasmid was constructed by inserting into the *Bam*HI-linearized YCpAJ152 plasmid (3) a 0.86-kb DNA fragment flanked by *Bam*HI restriction sites and spanning the first nine codons of *MEP2* plus 828 bp of upstream sequence. This DNA fragment was obtained by PCR amplification with the primers 5′-CGCCGGATCCGAATACAGATTGGATGCACTGG-3′ and 5′-GGCGGATCCGTAGGCGTACCTGTAAAATT-3′. The YCp*MEP3-lacZ* plasmid was constructed by inserting into *Bam*HI-linearized YCpAJ152 a 0.89-kb DNA fragment flanked by *Bam*HI restriction sites and covering the first nine codons of *MEP3* plus 866 bp of upstream sequence. This DNA fragment was obtained by PCR amplification with the primers 5′-CGCGGATCCCAAGATC AACGAGCCACAAGT-3′ and 5′-GGCGGATCCCATAGATGTCCGTCACC CCG-3′. The accuracy of the PCR DNA fragments used to construct *MEP2-lacZ* and *MEP3-lacZ* was verified by sequencing. The *MEP2* deletion plasmid (pAM204) was constructed by inserting into the *Hin*dIII- and *Eco*RI-cleaved pGEM7Zf$^+$ plasmid a 0.34-kb *Hin*dIII-*Hin*cII segment covering the 5′ flanking region of *MEP2* (positions −288 to +54) linked with a 0.46-kb *Hin*cII-*Eco*RI segment covering the 3′ *MEP2* region (positions +988 to +1444). Subsequently, a 2.7-kb *Bgl*II-*Bgl*II fragment carrying the selectable *LEU2* marker of pFL36 was blunt ended and inserted into the *Hin*cII site present in the *MEP2* sequences. Plasmids pFL36 and pFL38 have been previously described (8).

Construction of strains with *MEP* deletions. Construction of a *mep1*Δ *ura3* strain has been described previously (34). A *mep1*Δ *mep2*Δ *ura3* strain was isolated from a *mep1*Δ *leu2 ura3* strain by one-step replacement (41) of the *MEP2* gene (positions +54 to +988) with the *LEU2* marker. For this, strain 31000a was transformed with the *Hin*dIII-*Eco*RI fragment of plasmid pAM204. Among the $Leu2^+$ transformants, at least three (including strain AM2) harbored the *MEP2* replacement. A single *mep3*Δ *ura3* strain was constructed by the PCR-based gene disruption method (52). The *mep3*Δ*::KanMX2* heterologous module was synthesized by PCR amplification with the primers 5′-CGCTACG TATGATTGTCTATTACAATCAATGCAGGCTAGTTAAGGGTCCCAGC TGAAGCTTCGTACGC-3′ and 5′-CGCTCTAGAACTTCCGTTCAATGTGC CGACGAGCAGTTACTGGGGAGTTGCATAGGCCACTAGTGGATCTG-3′. The resulting *mep3*Δ*::KanMX2 ura3* strain was then crossed with the wild-type strain to yield strain 31011a. Except for the *mep1*Δ *ura3* and *mep3*Δ *ura3* strains, all *mep*Δ combinations were obtained by dissecting genetic crosses between strains AM2 and 31011a.

[^{14}C]methylammonium uptake, ammonium removal, and enzyme assays. Initial rates of [^{14}C]methylammonium (Amersham) uptake were measured as described for amino acids (21) with cells grown on minimal proline medium. For cation competition experiments, samples of cells grown on minimal proline medium were first filtered and washed with 20 mM sodium phosphate buffer (pH 6.1) plus 3% glucose and then resuspended in an equal volume of buffer (34). For the NH_4^+ removal assays, cells grown on proline medium were filtered, washed with proline medium, and resuspended in proline medium plus NH_4^+ at the appropriate concentration. At intervals, cells were removed from culture samples (1 ml) by filtration. The NH_4^+ content of the medium was determined by coupling with L-glutamate dehydrogenase (34, 51). β-Galactosidase assays were performed as described previously (3); enzyme activities are expressed as nanomoles of *o*-nitrophenol formed per minute per milligram of protein. Protein concentrations were measured by the method of Lowry et al. (31) with bovine serum albumin as the standard.

All assays were performed on cells having reached the state of balanced growth (54). The sole exception is for RNA extraction from cells grown on NH_4^+ at a limiting initial concentration (<1 mM) as the sole nitrogen source, in which case the collected cells were nitrogen starved, because NH_4^+ was most certainly totally consumed.

RESULTS

***MEP2* encodes a high-affinity NH_4^+ transporter homologous to Mep1p.** The *MEP2* gene was cloned by functional complementation of the *mep1-1 mep2-1* double mutant lacking both previously described NH_4^+ transport activities (14). The double mutant grows much more slowly than the wild type on media in which the sole nitrogen source is NH_4^+ at a low concentration (1 mM), while the corresponding single mutants (*mep1-1* and *mep2-1* mutants) grow almost normally under such conditions. The *mep1-1* mutation also confers resistance to the growth-inhibiting effects of toxic external concentrations of methylammonium (50 mM). Strain 26972c (*mep1-1 mep2-1 ura3*) was transformed with a genomic library constructed in a low-copy-number plasmid and representing the genome of wild-type strain Σ1278b. A plasmid (YCpAM21) carrying a 7-kb genomic insert was isolated for its ability to restore growth on 1 mM NH_4^+ without affecting resistance to methylammonium. This plasmid restored the ability of the *mep1-1 mep2-1* strain to take up [^{14}C]methylammonium at a high rate (Fig. 1A). The kinetic parameters of the corresponding activity were calculated from Lineweaver-Burk plots over the concentration range of 0.025 to 1 mM (Fig. 1B). The K_m (0.25 mM) and V_{max} (20 nmol/min/mg of protein) are identical to those previously measured for a *mep1-1* strain (14). Plasmid YCpAM21 also restored the ability of the *mep1-1 mep2-1* strain to remove NH_4^+ at a high rate from minimal proline medium containing 0.5 mM NH_4^+ (Fig. 1C). Ammonium ions competitively inhibited the [^{14}C]methylammonium uptake activity, with a K_i in the range of 1 to 2 μM (Fig. 1D). Furthermore, the uptake activity restored by the YCpAM21 plasmid appears to be highly NH_4^+ specific, as judged from competition experiments performed with mono- and bivalent cations (Table 2). Sequencing of the YCpAM21 genomic insert and subcloning experiments led to the isolation of a 2.4-kb *Bst*EII-*Hpa*I DNA fragment (accession number X83608) bearing a single, 1,497-bp open reading frame, which was sufficient to complement the *mep2-1* mutation. To definitively prove that this recombinant clone contains the *MEP2* gene, the corresponding locus was analyzed in the 26972c strain carrying the *mep2-1* allele. With oligonucleotide primers hybridizing, respectively, 0.83 kb upstream and 0.56 kb downstream of the coding region, PCR amplification performed on wild-type DNA generated an expected 2.9-kb DNA fragment, while a much shorter one (0.55 kb) was obtained from the 26972c strain. Cloning and sequencing of this 0.55-kb fragment revealed that a 2.33-kb region covering the entire coding region plus 0.44 kb of upstream and 0.39 kb of down-

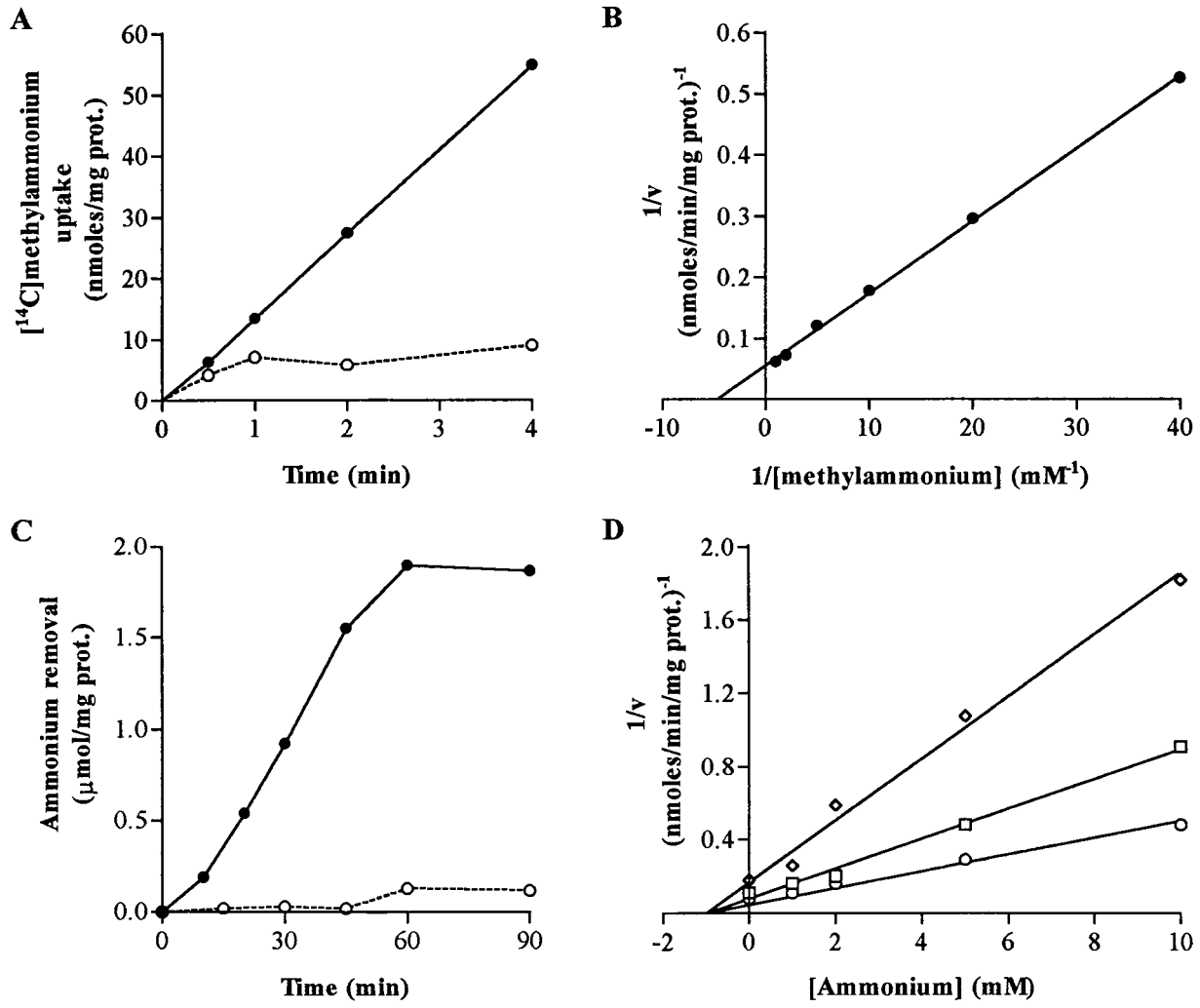


FIG. 1. Characterization of Mep2 transport activity. (A) Time course of [^{14}C]methylammonium accumulation (initial concentration, 0.5 mM) measured in cells grown on minimal proline medium. The strain was 26972c (*mep1-1 mep2-1 ura3*) transformed with pFL38 (○) or YCpAM21 (●). (B) Lineweaver-Burk plot of [^{14}C]methylammonium uptake into cells grown on minimal proline medium. The strain was 26972c (*mep1-1 mep2-1 ura3*) transformed with YCpAM21. (C) Time course of ammonium removal from minimal proline medium containing 0.5 mM ammonium. The strain was 26972c (*mep1-1 mep2-1 ura3*) transformed with pFL38 (○) or YCpAM21 (●). (D) Competitive inhibition of [^{14}C]methylammonium uptake by ammonium in cells grown on minimal proline medium. Measurements were performed in the presence of [^{14}C]methylammonium at 0.1 mM (◇), 0.25 mM (□), or 0.5 mM (○). The strain was 26972c (*mep1-1 mep2-1 ura3*) transformed with YCpAM21.

stream sequences has been deleted in the *mep2-1* strain. The deletion sites occur in 32- and 42-bp poly(T) sequences flanking the *MEP2* gene, suggesting that *MEP2* was naturally deleted by homologous recombination. The *MEP2* gene encodes a highly hydrophobic, 499-amino-acid protein (53.4 kDa) very similar in sequence to the Mep1p NH_4^+ transporter (41% identity) (Fig. 2). During systematic sequencing of chromosome XIV, the sequence of the *MEP2* gene (*YNL142w*) of strain S288c was determined (33). No differences between the two *MEP2* sequences were detected.

***MEP3* encodes a third, lower-affinity NH_4^+ transporter.** The *mep1-1 mep2-1* double mutant originally isolated by chemical mutagenesis (14) grows very slowly on NH_4^+ at a low concentration (1 mM). A yeast strain lacking the *MEP1* and *MEP2* genes was isolated from the *mep1*Δ *MEP2*$^+$ strain (34) by replacing the *MEP2* gene with *LEU2*. Unexpectedly, the resulting *mep1*Δ *mep2*Δ double mutant grew normally on 1 mM NH_4^+ (Fig. 3), suggesting the existence of another NH_4^+ transporter able to support growth on low-NH_4^+ medium. Accordingly, a *mep1*Δ *mep2*Δ strain can remove NH_4^+ (1 mM) from the medium much faster than the original *mep1-1 mep2-1* strain (Fig. 4). Hence, yeast cells must possess a third NH_4^+ transport system which for some reason is inactive in the initially isolated *mep1-1 mep2-1* strain. Database screening with the Mep1p sequence as the query sequence revealed a 3′-end *S. cerevisiae* cDNA sequence (accession number T38466) whose translation yields a short peptide highly similar to a C-terminal sequence conserved in Mep1p and Mep2p. An oligonucleotide defined on the basis of this cDNA sequence was used as a probe to screen a genomic library of strain Σ1278b. We thus isolated three partially overlapping DNA fragments, the shortest of which (YCpLEB5, 7 kb) was shown to hybridize with the probe in its central region. Sequencing of this region revealed the presence of a gene, *MEP3*, encoding a 489-amino-acid protein (53.7 kDa) highly similar in sequence to Mep1p (79% identity) and Mep2p (39% identity) (Fig. 2). Meanwhile, systematic sequencing of chromosome XVI also yielded a *MEP3* sequence (*YPR138c*) differing by a single nucleotide

TABLE 2. Inhibition of Mep2 uptake activity by monovalent and bivalent cations[a]

Added cation	Concn (mM)	Uptake activity (nmol min^{-1} mg of $protein^{-1}$)
None		30
NH_4^+	0.5	4.5
	10	3.6
Li^+	0.5	37
	10	30
K^+	0.5	36.5
	10	26
Rb^+	0.5	41
	10	33
Cs^+	0.5	38
	10	25
Mg^{2+}	0.5	40
	10	24
Ca^{2+}	0.5	29
	10	12

[a] Rates of uptake of 0.5 mM [^{14}C]methylammonium in *mep1-1 mep2-1* cells (strain 26972c) bearing the *MEP2* gene on a centromere-based plasmid (YCpAM21) were measured.

(T→A) from the *MEP3* sequence determined for strain Σ1278b (the difference causes a Phe→Tyr substitution at residue 39). The high similarity of the three Mep proteins strongly suggests that Mep3p is also an NH_4^+ transporter. To test this hypothesis, the PCR-based replacement method (52) was used to completely delete *MEP3* from the *mep1*Δ *mep2*Δ strain. Unlike the double-null strain, the triple-null mutant failed to grow on low-NH_4^+ medium (Fig. 3). Removal of 1 mM NH_4^+ from the medium was also abolished in this strain as compared to the case for the *mep1*Δ *mep2*Δ double mutant (Fig. 4). These data indicate that *MEP3* encodes another NH_4^+ transport protein. Experiments to determine why the *mep1-1 mep2-1* strain is deficient in Mep3p transport activity are in progress.

As previously shown, Mep1p and Mep2p behave like high-affinity NH_4^+ transporters. To determine the kinetic parameters of Mep3p, the initial rate of uptake of [^{14}C]methylammonium (0.5 to 2 mM) was first measured in the double (*mep1*Δ *mep2*Δ *MEP3*) and triple (*mep1*Δ *mep2*Δ *mep3*Δ) mutant strains grown on proline medium. At no substrate concentration tested did cells expressing Mep3p display a significantly higher initial methylammonium accumulation rate than the triple-*mep* strain. Since Mep1p and Mep2p have a higher affinity for NH_4^+ than for methylammonium, the absence of methylammonium uptake via Mep3p might be due to Mep3p being a much-lower-affinity transporter. This was confirmed in NH_4^+ removal assays, performed with Mep3p-expressing cells, where the initial external NH_4^+ concentration was varied from 0.5 to 4 mM (data not shown). The results showed that Mep3p has a lower affinity for NH_4^+ than Mep1p and Mep2p (K_m, ~1.4 to 2.1 mM). On the other hand, Mep3p's maximal rate of ammonium uptake (V_{max}, ~70 nmol/min/mg of protein) is higher than those of Mep1p and Mep2p.

Comparative phenotypic analysis of strains with different combinations of *MEP* gene deletions. To further compare the properties of Mep1p, Mep2p, and Mep3p, strains bearing *MEP* deletions in all combinations were constructed (Table 1). The rate of removal of NH_4^+ (1 mM) was first compared for strains expressing a single Mep protein (Fig. 4). All three double-*mep* strains incorporated NH_4^+ at a rate much higher than that of the triple-*mep* strain, confirming that each Mep protein can act individually as an NH_4^+ transporter. It is noteworthy that the activity displayed by wild-type cells did not equal the sum of the three individual Mep activities. Instead, Mep1p-expressing cells displayed a rate of NH_4^+ retrieval from the medium similar to that measured for the wild-type, while Mep2p- and

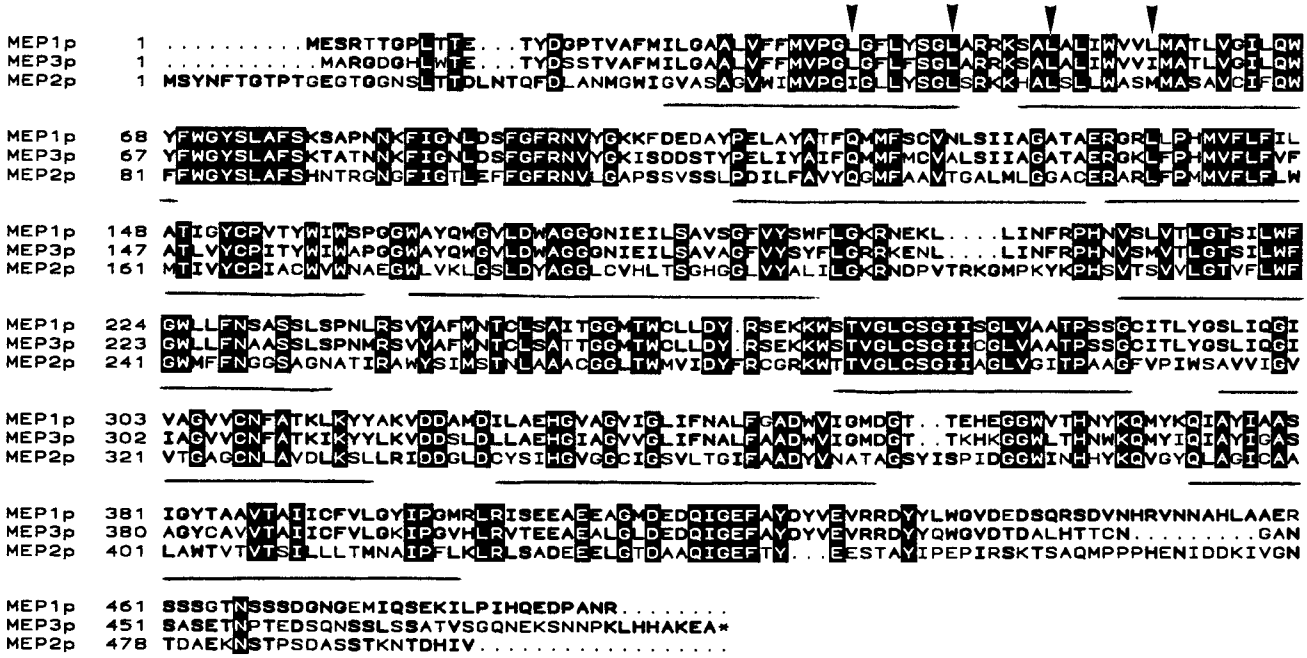


FIG. 2. Sequence alignment of Mep1p, Mep2p, and Mep3p of *S. cerevisiae*. The amino acid sequences were aligned by using PILEUP (13). Identical residues are in black boxes. Similar residues are in boldface. The transmembrane segments predicted by the TMAP algorithm (38) on the multiple sequence alignment are underlined. The four residues of the putative leucine zipper are marked by arrowheads.

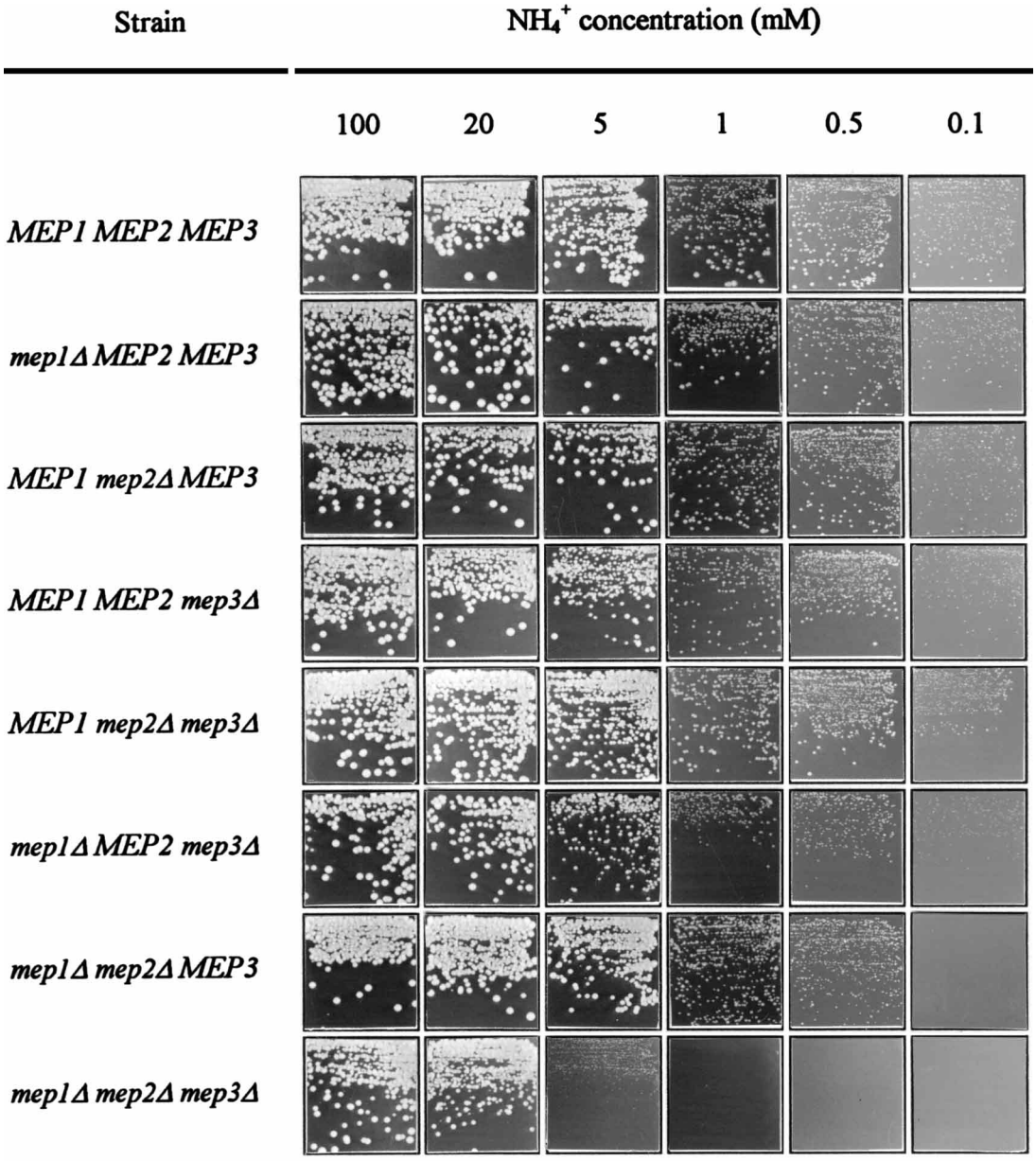


FIG. 3. Growth test on minimal medium containing various NH_4^+ concentrations as the sole nitrogen source. The strains were 23344c (*ura3*), AM1 (*mep1*Δ *ura3*), 31021d (*mep2*Δ *ura3*), 31011a (*mep3*Δ *ura3*), 31018b (*mep2*Δ *mep3*Δ *ura3*), 31022a (*mep1*Δ *mep3*Δ *ura3*), 31021c (*mep1*Δ *mep2*Δ *ura3*), and 31019b (*mep1*Δ *mep2*Δ *mep3*Δ *ura3*). All strains were transformed with pFL38.

Mep3p-expressing cells displayed lower, roughly similar, NH_4^+ retrieval rates.

Growth of all *mep* mutants on media containing various NH_4^+ concentrations as the sole nitrogen source was also tested (Fig. 3). A strain lacking the three Mep proteins cannot grow on media containing less than 5 mM NH_4^+ but grows normally on high-NH_4^+ (>20 mM) media. Compared to the wild-type strain, cells with a single *MEP* gene deletion are not significantly affected for growth on all NH_4^+ concentrations tested. In contrast to the triple mutant, cells expressing a single Mep protein are able to grow on NH_4^+ at low concentration (≤5 mM). However, under the latter conditions, the three double mutants display growth differences which may be interpreted in terms of kinetic properties of individual NH_4^+ transporters. The Mep1p protein, which displays a high affinity and a high V_{max}, supports optimal growth on all low NH_4^+ concentrations. Mep2p, the transporter with highest affinity and lowest V_{max}, supports a slower growth than Mep1p. In this case, the low V_{max} appears to be the limiting parameter. On the other hand, Mep3p, the transporter with lowest affinity and highest V_{max}, supports optimal growth on saturing NH_4^+ concentrations but is most sensitive to lowering of the NH_4^+ concentration below 1 mM.

The Mep transporters are also required for NH_4^+ retention. Previous studies have shown that some transport proteins are required not only for uptake of compounds available in the medium but also for retention of intracellular metabolites which under certain conditions tend to leak out of the cells.

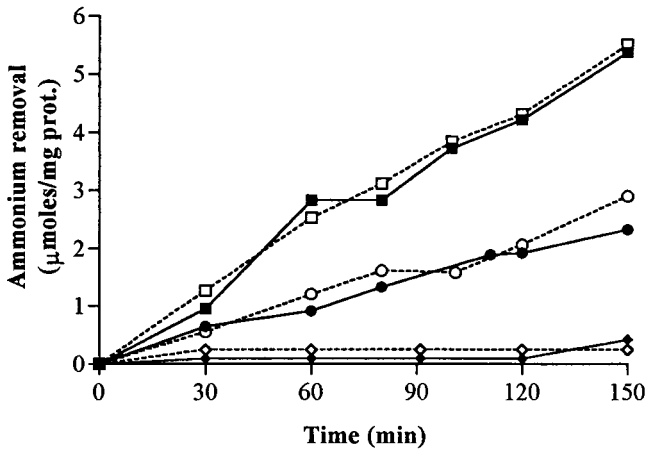


FIG. 4. Time course of ammonium removal from minimal proline medium containing 1 mM ammonium. The strains were 23344c (*ura3*) (■), 31018b (*mep2*Δ *mep3*Δ *ura3*) (□), 31022a (*mep1*Δ *mep3*Δ *ura3*) (●), 31021c (*mep1*Δ *mep2*Δ *ura3*) (○), 31019b (*mep1*Δ *mep2*Δ *mep3*Δ *ura3*) (◆), and 26972c (*mep1-1 mep2-1 ura3*) (◇). All strains were transformed with pFL38.

This has been well illustrated in feeding experiments which allow the study of the excretion phenomenon (18). For instance, a mutant strain auxotrophic for arginine (receptor cells) could be arginine fed by a mutant strain lacking arginine uptake activity (donor cells), while the wild-type strain was unable to feed the receptor cells. Hence, arginine permease activity is somehow needed for arginine retention inside cells. While characterizing the Mep proteins, we likewise made observations indicating that NH_4^+ transporters play a role in the retention of NH_4^+ within the cells. In one experiment, we tested the abilities of different strains to feed, on a medium containing arginine (0.05%) as the sole nitrogen source, a receptor strain lacking arginine uptake activity (*gap1-1 can1-1* strain) which grows slowly on that medium (Fig. 5). When wild-type donor cells were used, no feeding of the *gap1-1 can1-1* receptor cells was detected after a 3-day incubation (Fig. 5A). In contrast, the triple-*mep* strain was clearly able to feed the cells (Fig. 5E), so it must excrete into the medium a nitrogen source that can be taken up by cells lacking the general amino acid permease Gap1p and the specific arginine permease Can1p. That this nitrogen source is NH_4^+ is suggested by the fact that no feeding was detected when the donor cells expressed a single high-affinity NH_4^+ transporter (Mep1p or Mep2p) (Fig. 5B and C), while donor cells expressing only the low-affinity NH_4^+ transporter Mep3p gave rise to a detectable feeding of the receptor cells (Fig. 5D). After a longer incubation period (5 days), barely detectable feeding of the *gap1-1 can1-1* receptor cells was also observed with donor cells expressing either Mep1p or Mep2p alone and even with the wild-type strain (data not shown). Similar experiments were carried out with media containing proline, GABA, or glutamate as the sole nitrogen source, using, respectively, mutant strains lacking proline, GABA, or glutamate uptake activity as receptor cells. With these nitrogen sources, none of the donor strains could feed the receptor strains (data not shown). These data suggest that during growth on a medium containing arginine, a nitrogen source whose catabolism produces both glutamate and NH_4^+, wild-type cells excrete NH_4^+ ions which are then taken up by the high-affinity permeases Mep1p and Mep2p and less efficiently by Mep3p. As further evidence for the importance of the Mep proteins in NH_4^+ retention, cells lacking both Mep1p and Mep2p grow more slowly than the wild-type strain on a medium containing urea, a poor nitrogen source which is entirely converted to NH_4^+ (data not shown).

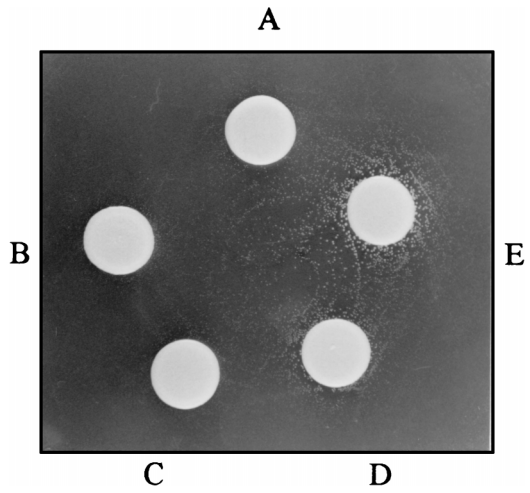


FIG. 5. Feeding of a strain lacking arginine permease by a mutant lacking ammonium permease. The receptor strain, 21983c (*gap1-1 can1-1 ura3*), was plated on arginine (0.05%) minimal medium to a low population density so as to give distinct colonies when growing. Donor strains 23344c (*ura3*) (A), 31018b (*mep2*Δ *mep3*Δ *ura3*) (B), 31022a (*mep1*Δ *mep3*Δ *ura3*) (C), 31021c (*mep1*Δ *mep2*Δ *ura3*) (D), and 31019b (*mep1*Δ *mep2*Δ *mep3*Δ *ura3*) (E) were dropped on the plate to a high population density.

Structural conservation of NH_4^+ transport proteins in yeast and other organisms. The hydropathy profiles (28) of Mep2p and Mep3p are very similar to that of Mep1p (34) (data not shown). Each of the three proteins consists of a central hydrophobic core made of 10 to 12 transmembrane domains flanked by hydrophilic sequences. Several algorithms, including TMAP (38) and PHDhtm (40), predict that the central hydrophobic region contains 10 transmembrane segments. The three amino acid sequences differ mostly at their hydrophilic N and C termini (Fig. 2), but the 40-amino-acid region immediately C terminal to the last predicted transmembrane domain is also highly conserved. Like Mep1p, Mep3p contains a putative leucine zipper motif consisting of a heptad repeat of four leucine or isoleucine residues which is also located in a region spanning parts of the first two putative transmembrane domains (Fig. 2). This motif also occurs in Mep2p, except for the substitution of methionine for the fourth leucine or isoleucine residue. Leucine zipper motifs have been observed at similar locations in several vertebrate and fungal glucose transporters (7, 53). It is still unknown whether these motifs have any function in transport proteins, but it has been suggested that they might be involved in oligomerization (7).

Analysis of the nonredundant EMBL/GenBank sequence databases with the BLAST algorithm (1) revealed no fewer than 21 Mep-homologous proteins (Fig. 6). Most of these sequence determinations resulted from genome sequencing efforts. Except for the recently characterized (methyl)ammonium permease of *Corynebacterium glutamicum* (47), the 10 deduced protein sequences identified so far in bacterial organisms are of unknown function, including three in a *Synechocystis* sp. (26) and two in the archaebacterium *Methanococcus jannaschii* (10). No Mep-homologous protein was found by examining the complete genomic sequences of *Haemophilus influenzae* and *Mycobacterium genitalium*, two bacteria whose natural environment is human tissues. Several Mep-homologous proteins are encountered in plants (16), such as the characterized NH_4^+ transporters of *Arabidopsis thaliana* and *Lyco-*

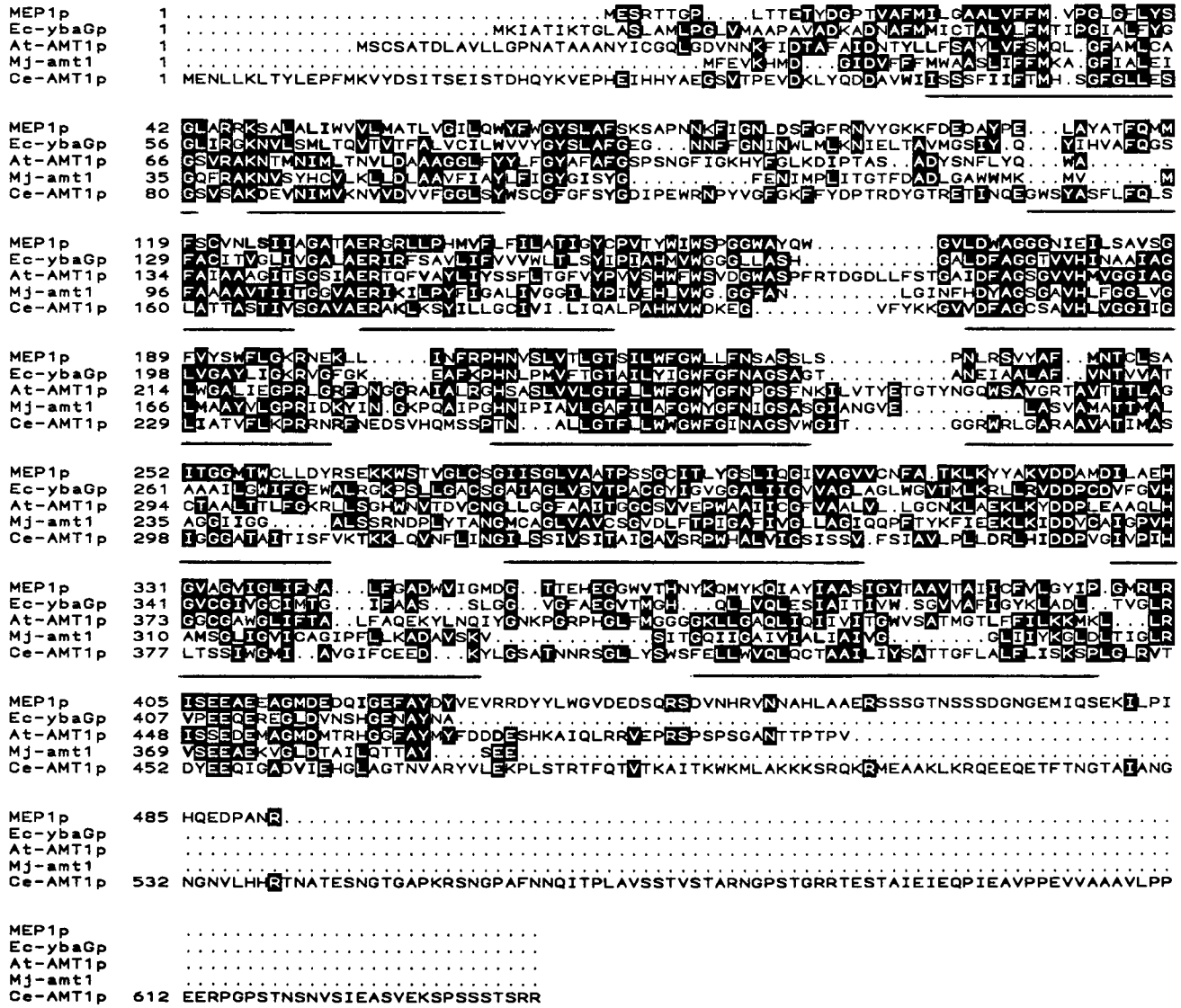
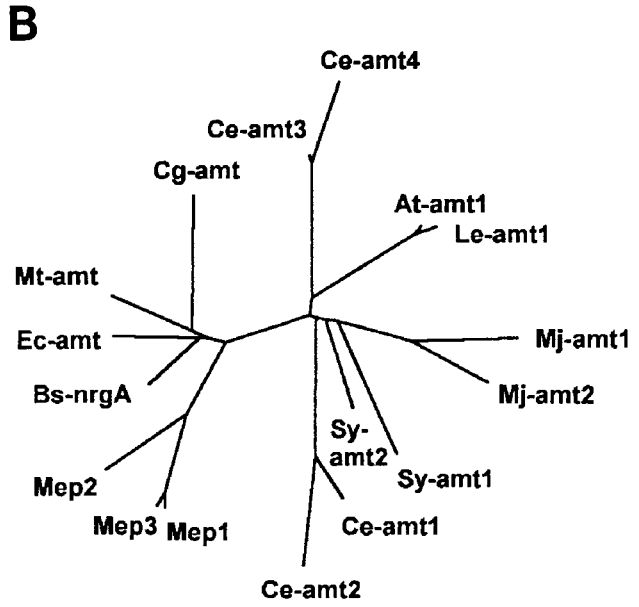


FIG. 6. (A) Conservation of the Mep/Amt family in eubacteria, archaebacteria, plants, and animals. The amino acid sequences were aligned by using PILEUP (13). Identical residues are in black boxes. Similar residues are in boldface. MEP1p of *S. cerevisiae* (34) is aligned with a protein of *E. coli* (Ec), ybaGp (9); a protein of *A. thaliana* (At), AMT1p (35); a protein of *Methanococcus jannaschii* (Mj), amt1 (10); and a protein of *Caenorhabditis elegans* (Ce), AMT1p (55). The transmembrane segments predicted by the TMAP algorithm (38) on the multiple sequence alignment are underlined. (B) Phylogenetic tree of ammonium transporters and similar proteins of unknown function. The amino acid sequences were aligned by using PILEUP (13). The PROTDIST algorithm (15) was used to compute a distance matrix based on the Dayhoff PAM matrix. The calculation was restricted to the region of the sequence alignment that corresponds to the conserved central hydrophobic cores of the proteins (residues 90 to 610). The phylogenetic tree was constructed by the neighbor-joining method (43) and drawn with DRAWTREE (15). Bs, *Bacillus subtilis*; Cg, *Corynebacterium glutamicum*; Mt, *Mycobacterium tuberculosis*; Sy, *Synechocystis* sp.; Le, *L. esculentum.*

persicon esculentum (29, 35). Finally, four Mep homologs were found in the roughly half-sequenced genome of the nematode *Caenorhabditis elegans* (55). These data reinforce the previous conclusion that the Mep/Amt protein family is widely conserved (34, 35). They further suggest that families of NH_4^+ transporters exist in organisms other than yeast.

The *MEP1*, *MEP2*, and *MEP3* genes are expressed at different levels and are subject to nitrogen control. The physiological roles of Mep1p, Mep2p, and Mep3p are to scavenge external ammonium for use as a nitrogen source and to incorporate ammonium which under some circumstances leaks out of the cells. Previous experiments have shown that expression of *MEP1* is subject to nitrogen catabolite repression (34). We used Northern analysis and *lacZ* fusions to determine whether expression of *MEP2*, *MEP3*, or both also responds to nitrogen control in wild-type cells.

Northern analysis revealed a single band hybridizing with the probe used to detect *MEP2* transcripts and corresponding to the expected size of 1.6 kb. The intensity of the signal varied markedly according to the nitrogen source available in the medium (Fig. 7). *MEP2* RNA accumulated most abundantly in cells grown on NH_4^+ at a limiting concentration or on a poor



Organism	Acc. number
Eubacteria	
Bacillus subtilis	*L03216*
Corynebacterium glutamicum	*X93513*
Escherichia coli	*U40429*
Mycobacterium leprae	*L78818*
Mycobacterium tuberculosis	*Z74697*
Rhodobacter capsulatus	*X12359*
Synechocystis sp.	*D64006 (amt1)* *D64004 (amt2)* *D90901 (amt3)*
Archaebacteria	
Methanococcus jannaschii	*U67463(amt1)* *U67574 (amt2)*
Fungi	
Saccharomyces cerevisiae	*X77608 (Mep1)* *X83608 (Mep2)* *U40829 (Mep3)*
Plants	
Arabidopsis thaliana	*X75879*
Lycopersicon esculentum	*X92854*
Animals	
Caenorhabditis elegans	*Z66498 (amt1)* *Z70308 (amt2)* *U53338 (amt3,-4)*

FIG. 6—*Continued.*

nitrogen source like proline. Glutamate-grown cells displayed a relatively large amount of *MEP2* RNA, while cells grown on good nitrogen sources like glutamine or a high concentration of NH_4^+ produced a weaker signal. Like *MEP1* expression, *MEP2* expression is thus subject to nitrogen catabolite repression, in keeping with the measurements of Mep2p-dependent methylammonium uptake activity (14, 34). In proline-grown cells, however, *MEP2* RNA was found to accumulate to levels roughly 20 times higher than those of *MEP1* RNA (Fig. 7). This observation was confirmed by comparing expression levels of the *lacZ* gene under the control of the *MEP1* and *MEP2* promoter regions (Table 3); in proline- or glutamate-grown cells, the *MEP2-lacZ* gene was expressed to levels 15 to 20 times higher than those of the *MEP1-lacZ* gene. On favored nitrogen sources (0.1% glutamine, 20 mM NH_4^+, or both), expression of both genes was down-regulated. *MEP1-lacZ* expression and *MEP3-lacZ* expression were similar, but the latter was two times lower in proline-grown cells (Table 3). Hence, all three *MEP* genes are subject to nitrogen catabolite repression, but *MEP2* is expressed to a much higher level than *MEP1* and *MEP3*. Consistently, the codon bias index (6) is higher for *MEP2* (0.281) than for *MEP1* (0.081) or *MEP3* (0.141).

Differential control of *MEP1*, *MEP2*, and *MEP3* transcription by the general nitrogen regulatory factors Gln3p and Nil1p. Nitrogen-regulated transcription in *S. cerevisiae* is mediated by at least two general positive GATA family factors, namely, Gln3p and Nil1p/Gat1p (11, 32, 49, 50). The GATA proteins are conserved among eukaryotes and share a highly conserved DNA-binding domain that promotes recognition of 5′-GAT(A/T)A-3′ core sequences. Typically, the upstream region of a yeast gene responding to nitrogen control contains several 5′-GAT(A/T)A-3′ sequences acting as upstream activating sequences sensitive to nitrogen catabolite repression. The GATA factors Gln3p and Nil1p contribute to various degrees to transcriptional activation mediated by these upstream 5′-GAT(A/T)A-3′ sequences. As expected, the *MEP1*, *MEP2*, and *MEP3* promoter regions contain several 5′-GAT (A/T)A-3′ core sequences that are likely involved in nitrogen-regulated transcription (Fig. 8). To investigate the role of Gln3p and Nil1p in expression of the three *MEP* genes, we introduced the corresponding *lacZ* fusions into mutant cells lacking the *GLN3* gene, the *NIL1* gene, or both and monitored their expression in cells grown on various nitrogen sources (Table 3).

MEP2-lacZ gene expression is highest in proline-grown wild-type cells. Under these growth conditions, it is almost unaltered in *nil1*Δ cells, reduced twofold in *gln3*Δ cells, but totally abolished in *nil1*Δ *gln3*Δ double-mutant cells. Hence, at least one positive GATA factor (Nil1p or Gln3p) is required for high-level expression of *MEP2* on proline medium. On glutamate medium, in contrast, expression of *MEP2-lacZ* is almost totally abolished in *gln3*Δ cells. Gln3p thus appears to be the main factor promoting expression of *MEP2-lacZ* in glutamate-grown wild-type cells. This is consistent with the view that Nil1p is inhibited by a high intracellular concentration of glutamate (50). Interestingly, the basal *MEP2-lacZ* expression

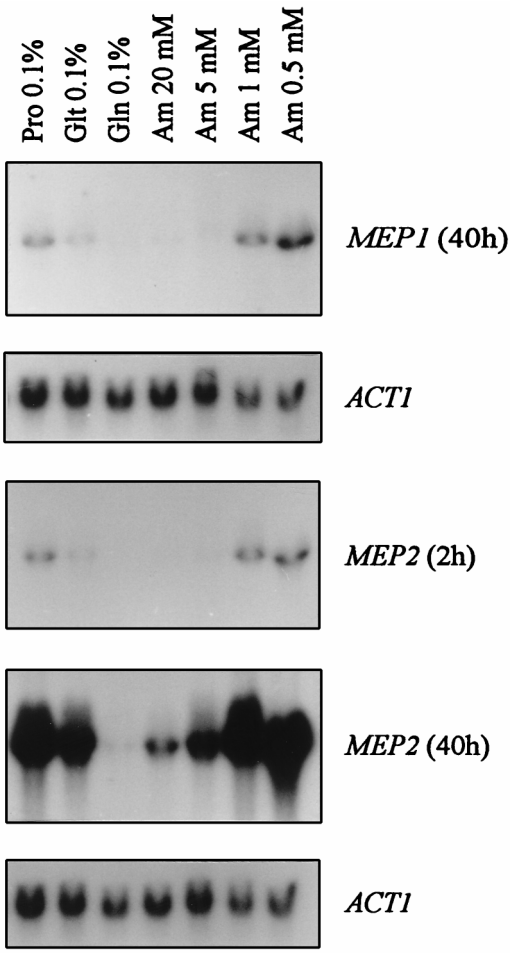


FIG. 7. Parallel Northern blot analysis of *MEP1* and *MEP2* transcripts in the wild-type strain Σ1278b. Total RNA was extracted from cells grown on minimal medium containing different nitrogen sources. Pro, proline; Glt, glutamate; Gln, glutamine; Am, ammonium ions. RNA was hybridized with randomly [α-^{32}P]dATP-primed probes: a 1.86-kb *Pst*I-*Xho*I DNA fragment containing the *MEP1* gene and a 1.86-kb *Hin*dIII-*Ssp*I DNA fragment containing the *MEP2* gene. Actin RNA (*ACT1*) was assayed to determine the loading efficiency. For comparison, the periods of autoradiograph exposure are indicated.

observed in wild-type cells grown with a high NH_4^+ concentration (20 mM) requires both Nil1p and Gln3p, since expression is abolished in single *gln3*Δ and *nil1*Δ mutant strains. The two positively acting GATA factors thus seem to be unable to compensate for each other's absence under these conditions, although they seem to do so in proline-grown cells.

The results obtained with *MEP1-lacZ* and *MEP3-lacZ* differed from those described above. Whether the nitrogen source was proline, glutamate, or NH_4^+, expression of *MEP1-lacZ* and *MEP3-lacZ* was abolished in cells lacking Gln3p. Surprisingly, deletion of *NIL1* resulted in ~7-fold-higher expression of *MEP1-lacZ* and *MEP3-lacZ* in proline- and glutamate-grown cells but not in cells grown on glutamine or high-NH_4^+ medium. Nil1p thus appears to down-regulate Gln3p-dependent expression of *MEP1* and *MEP3* in glutamate- and proline-grown cells. To our knowledge, this is the first evidence that Nil1p can act directly or indirectly as a negative transcription factor.

In conclusion, both Gln3p and Nil1p can promote high-level expression of *MEP2*, but the contribution of each factor depends on the nitrogen source(s) available. In the case of *MEP1* and *MEP3*, Gln3p appears to be the main activator, but a Nil1p-dependent mechanism maintains expression of both genes at a relatively low level.

DISCUSSION

This study shows that *S. cerevisiae* possesses three ammonium transporters, Mep1p, Mep2p, and Mep3p. All three proteins are highly similar in sequence, with Mep1p and Mep3p being more closely related. Apart from the three *MEP* genes, the completely sequenced genome of *S. cerevisiae* contains no other gene encoding a Mep homolog. This yeast might, however, possess additional NH_4^+ transport systems unrelated to the Mep proteins. Consistent with this view is the ability of the *mep1*Δ *mep2*Δ *mep3*Δ triple mutant to grow on a buffered medium (pH 6.1) in which the sole nitrogen source is NH_4^+ at a high concentration (>20 mM), although it cannot grow on low-NH_4^+ media. We cannot rule out, however, the possibility that NH_4^+ at a high concentration might be taken up into cells by simple diffusion.

S. cerevisiae has developed a multiplicity of NH_4^+ transporters displaying different but somewhat overlapping kinetic properties. As previously reported (14), the Mep2p transporter shows the highest affinity for NH_4^+ (1 to 2 μM). It is followed by Mep1p (5 to 10 μM) (14, 34) and finally by Mep3p, whose affinity is much lower (1.4 to 2.1 mM). While Mep1p and Mep3p display very different K_m values, they have about 79% amino acid identity over their entire lengths. This homology reaches 86% if only the predicted membrane-spanning domains are compared. Maximal uptake rates mediated by the individual Mep proteins were measured in cells grown on proline medium, i.e., under conditions where expression of all three *MEP* genes is highest. Under these conditions, Mep3p displays the highest V_{max} (~70 nmol/min/mg of protein), followed by Mep1p and finally Mep2p (40 and 20 nmol/min/mg of protein, respectively). Under the same conditions, *MEP2* expression is about 20 times higher than *MEP1* expression, with the latter being 2 times higher than *MEP3* expression. If these differences in gene expression reflect the actual relative amounts of the active transporters, then in terms of the maximum velocity per active transporter, the three proteins must differ much more markedly than the apparent V_{max} values show.

The main physiological function of the Mep proteins is to scavenge NH_4^+ from the medium for use as a nitrogen source. Growth tests have shown that Mep1p, the transporter with high affinity and high V_{max}, supports optimal growth on all low NH_4^+ concentrations tested (0.1 to 5 mM). Mep2p, which displays the lowest V_{max}, supports a slower growth than Mep1p on NH_4^+ concentrations higher than 0.1 mM, whereas Mep3p, which displays the lowest affinity, is most sensitive to lowering of the NH_4^+ concentration below 1 mM. Another physiological role of these NH_4^+ transporters is highlighted by the feeding experiments, which suggest that they are also required for NH_4^+ retention inside cells during growth on at least some nitrogen sources other than NH_4^+. In this role of taking up NH_4^+ that tends to leak out of the cells, the high-affinity transporters Mep1p and Mep2p are the most effective.

The fact that each *MEP* gene alone gives rise to a specific NH_4^+ transport activity provides the best evidence that the Mep proteins are indeed NH_4^+ transporters. Yet this does not rule out a regulatory function for at least some Mep proteins. It has recently been reported, for instance, that the Mep proteins might also act as receptors involved in the development of pseudohyphal growth in response to nitrogen starvation (30, 42). Also pertinent is the role of NH_4^+ transporters in trigger-

TABLE 3. Nitrogen-regulated transcription of the *MEP1*, *MEP2*, and *MEP3* genes[a]

Nitrogen source (concn)	β-Galactosidase activity (nmol min^{-1} mg of $protein^{-1}$) with the following fusion gene and strain:											
	MEP1-lacZ				*MEP2-lacZ*				*MEP3-lacZ*			
	Wild type	*gln3*Δ mutant	*nil1*Δ mutant	*gln3*Δ *nil1*Δ mutant	Wild type	*gln3*Δ mutant	*nil1*Δ mutant	*gln3*Δ *nil1*Δ mutant	Wild type	*gln3*Δ mutant	*nil1*Δ mutant	*gln3*Δ *nil1*Δ mutant
Proline (0.1%)	91	7	603	3	2,103	1,209	1,836	≤1	38	12	238	6
Glutamate (0.1%)	49	6	319	3	754	28	758	≤1	63	5	328	7
Ammonium (20 mM)	25	≤1	26	≤1	181	7	5	≤1	28	≤1	21	2
Glutamine (0.1%)	3	≤1	2	≤1	10	≤1	6	≤1	8	2	9	2
Ammonium (20 mM) + glutamine (0.1%)	≤1	≤1	≤1	≤1	≤1	≤1	4	≤1	5	≤1	8	2

[a] Low-copy-number plasmids bearing the indicated *lacZ* fusion genes were introduced into the indicated strains. Transformed cells were grown on the indicated sole nitrogen sources.

ing NH_4^+-induced inactivation and repression of enzymes and permeases involved in the use of secondary nitrogen sources. Several studies have proposed that Snf3p, a hexose permease-like protein, might play a regulatory role in glucose transport (7, 27, 37). More recently, the isolation of dominant mutations in *SNF3* and *RGT2* has shown that the hexose permease-like proteins encoded by these genes play a determining role as glucose sensors involved in transcriptional induction of *HXT* genes in response to low (*SNF3*) or high (*RGT2*) external glucose concentrations (36). It is tempting to hypothesize that similar sensors also influence the control of nitrogen metabolism.

Each *MEP* gene possesses several 5′-GAT(A/T)A-3′ core sequences in its promoter region and displays the expression profile typical of genes subject to nitrogen catabolite repression. Accordingly, the GATA factors Gln3p and Nil1p, which are involved in nitrogen-regulated transcription, control the expression of all three *MEP* genes. Recent studies have shown that the relative contribution of each positive factor to transcriptional activation depends on the nitrogen source and is gene specific; both of these features are likely determined by the specific arrangement of multiple upstream 5′-GAT(A/T)A-3′ core sequences (11, 32, 49, 50). In this respect, on proline and glutamate media, the *MEP2* expression profile resembles that described for the *GAP1* gene, encoding the general amino acid permease (11, 32): in proline-grown cells, both Gln3p and Nil1p activate transcription and each can largely compensate for the other's absence, while in glutamate-grown cells, Gln3p is the sole positive GATA factor essential to *MEP2* expression, suggesting that Nil1p is inactive under these conditions. In contrast to the case for *GAP1*, however, residual *MEP2* expression in NH_4^+-grown cells seems to require the simultaneous presence of both factors. In the case of the *MEP1* and *MEP3* genes, Gln3p is essential to transcription on all tested media, but a Nil1p-dependent mechanism maintains expression of both genes at a relatively low level during growth on a poor nitrogen source. Unlike Nil1p-mediated activation of the transcription of *MEP2* and other genes, this negative effect of Nil1p on *MEP1* and *MEP3* transcription is also visible in the presence of glutamate. The gene-specific character of Nil1p's positive

```
-900 TGAAAACTATACTATTCAATACATAGAACGTAACACAATGGCGTCCCCTCAAAAGTGTACACTTCTTCAAGATGTTACTGCGGCTACATGCAACACCCCTAGAGCTATTTGTAAGGGAAG
-780 ACAAGATCTCAAGTGTTACCACAGCCCGTAATTGAGTTCGGCACTATTCGAGCGCACAAAAAATCCCGTACTGTTTTTTTTCAGATAAGAAAGACTGAGCCTATATAAACACTACCTGAA
-660 CCATTTTTAAGATAAAAGTTATATACGATAGAGTTATCCCCTCTTAATAATTGATCAAGAACCTCTGAAGCACAAAAGGGAGCATAAGAGCACTTAGGGACAAACGTATTTCTTTCCCCC
-540 ACTCCCCTTTTTCCCTTTGCGATGCACATCCATAACTTGTACTTGTGTCTTGACATTCTTCAAAATCAACATGGCGATGCTCTTCTACGGTTGTCCTTTGCCACGAACTTTGTTTTGGCGC
-420 CTCCCCTCCCTTTGTCAGGCTGAGTGCAAAAGAAAAGGCCCTGGTTGCTTATCTCTGCGGACACGCGCCCCTCTTATTGGATGAGACATCTCAGAGATTGCGATAACGATAAGATTCACA
-300 ATTGCTACAGACACCCTTGTGTGACATCTTATGGCTATGTAAGCACAGAAGAGAAGAAGAAAGAAATGACAAAGTATTGATGGAAGATGTTTTTCCTGAAAAAAAAAAAAAAAAAAAATC
-180 GGAAAAGTGGTGTAGGCTTCTATAGCACCTTCTGCTTACCAGTCGATAATGCAGTTCCAGAATAAGATACTTAAAAGGTAGTTTATTTATATAGGCGCTCTTGTAACTTGTTTAGGCTTA
 -60 TGATCAGAGAATTATTTTAGTAAACATAACTGCAGTACGGATTCTTATACTGTAGAAACTATG -- MEP1

-900 TCTAACTCTATGCTCTTTTTGGCGTCTGAATGAGCCATAACGAGGGAAATAACTGTATCAGGAATACTAACAGAATACAGATTGGATGCACTGGTGCTGGTGAGTTTTCTTTTTCTGATA
-780 TTATTGGATAATATTTTCAGCTATAGACTATCATCATTCAGCATTGGTCACGCTAAAATATACGTTCCATGCATACGTGATAGCCTACTCGGTTGTCAACTTCATTTTATAGGCTGCCTG
-660 GTCACCGATTACCGCCCCGCTCAGCAGGACGGTTTAAGCTTCTGTTCTTGGAAACGACGTAATTCATGTCGGCCATCGAAATCATTCCTGGCTTTGTAATAGTCATAATGAACAAGGAAT
-540 GCTGTCTTGGGTTTTTTTCCCTTTGCGAATACCCGGGCTGCCAAAATAGAAAAGGAACAAATGGTAGGTCTTTAAGATAAGAAAGATAAGATAAGACATCTTTTTTTTTTTTTTTTTTT
-420 TTTTTTTTTTTTCCGATAACAAAGAAGAATCCTTCGGTCTCTTCTTACTGCTGTTACTGCTTATCAAGAGTGTCTGGTAATAGTCAAAGCCAGATAAGATAAGAAATGTAAAATCGACTA
-300 TTTCCCTCGTAAAAAGCTTCAATTGCTCACGCATCGACGAATACTAAGCGGCGTGAAGACCCTGTGCCTTATCAATGAAGTATTCAGTACAAGAGGTGTTCATTCCGAGCCACTTTTCTG
-180 CACCATATATAAGCAGTAGTTACACATGTGCTAACCAAACATCAGTGGGTAGTAATCATTCGTGCTTTCACGTCCTTTCAAATTAAATATCCTTCTTAAATAATAAGTCTGCACAGAGCT
 -60 TTGAAAGAAGTTTAGTTTTGTAATATATCATACTTAATATATTACAATACAATATCAACAATG -- MEP2

-900 TCTAGATTTCTGAACAAGGGCATTAATGTTAAATGCAAGATCAACGAGCCACAAGTACTCTCGGATAATTTAGAGGAATTACGCGTTGCATTAAACGGTGGCGACAAATATAAACTAGTC
-780 TCACGGAAGTTAGATGTTGAATCTAAGAGGAATTTTGTGAAGGAATATATCAGCGATCAACGTAAAAAGAGGAAGTAGAAATACTTATGAAGGCATGCATGCATGCATACATATTTATAT
-660 ATATATGTCTTTAGGAGTATGTATTGCATTTAATCGAAACAAACAAGAAAAAGAAGAGTAGATGTGCCCGTTTCTGTCACTCCTATTTACTGACACTTTCTTGACCTACCTCAAACCAGT
-540 TCCAGTTCCGGCAAAAAGTGCCGCGCGGCGGAGAAACATCTCCACCTTATCTCGGCGCCAAATCCTTATCTCTCGTAGCTGGTTTGCCCGCGATAAGGCGGGCGAGTTATTTTGAAGTTT
-420 TCCATAAACTGGTTTTCCATCTCGAGGTTTTTCCTCGCTTTCCACGCTATGACCCTTTTAGTTAAGGTACCCGATGGCATACTTTATATTATATATATATGTTAAGTTAATATGTTT
-300 TAGCAGATTTGATATGCTGATATGCAGCACGGACTTTCCCTCTCCTTGTCTTATCGCATCTTATCGCAACAATTTGATAGATATCTTCTCCCTTTCCTATCTTGTAGAATAAGGTTGTGT
-180 GCTTTGAGTCTGATAGCCGTCTTCTTTCGGTCGCTTCTTCTCTCTTTTGGTTCTTTGATTGTCTATTACAATCAATGCAGGCTAGTTAAGGGTCCAATCACTTTTGAAATTGTTTTGTAA
 -60 AAAGCGAAGGCATTTTTTTTTTAGAAGATACAATTGAAAACATATAGATTTAGAGTTCACATG -- MEP3
```

FIG. 8. Sequences of the *MEP1*, *MEP2*, and *MEP3* promoter regions. Numbers on the left indicate the nucleotide position relative to the A of the putative ATG translational start codon. The 5′-GAT(A/T)A-3′ core sequences are in boldface and underlined.

and negative effects on transcription suggests that these effects are also determined by specific arrangements of upstream 5′-GAT(A/T)A-3′ core sequences. The physiological meaning of this direct or indirect Nil1p-dependent repression of *MEP1* and *MEP3* remains unclear. It is noteworthy that a negative effect of Gln3p on activation of the *GAP1* gene by Nil1p has been described (48, 50).

The data obtained by screening sequence databases for Mep homologs suggest that families of NH_4^+ transporters exist in other organisms as well. This seems to be true not only for other unicellular organisms, such as the bacteria *Methanococcus jannaschii* and *Synechocystis* sp., but also for plants and animals. Our observation that the nematode *Caenorhabditis elegans* has at least four genes encoding such proteins is particularly relevant, since the molecular study of specific NH_4^+ transporters in animals is a heretofore unexplored area.

ACKNOWLEDGMENTS

We thank Antonio Urrestarazu for helpful discussions. We thank Doris Rentsch and Jean-Yves Springael for comments on the manuscript. We are also grateful to Catherine Lebeau for her contribution in characterizing the *MEP3* gene.

This work was supported by the Fund for Medical Scientific Research (Belgium) (FRSM 3.4602.94). A.-M.M. is recipient of an F.R.I.A. (Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture) predoctoral fellowship.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **André, B.** 1995. An overview of membrane transport proteins in *Saccharomyces cerevisiae*. Yeast **11:**1575–1611.
3. **André, B., C. Hein, M. Grenson, and J.-C. Jauniaux.** 1993. Cloning and expression of the *UGA4* gene coding for the inducible GABA-specific transport protein of *Saccharomyces cerevisiae*. Mol. Gen. Genet. **237:**17–25.
4. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. Wiley Interscience, New York, N.Y.
5. **Béchet, J., M. Grenson, and J.-M. Wiame.** 1970. Mutations affecting the repressibility of arginine biosynthetic enzymes in *Saccharomyces cerevisiae*. Eur. J. Biochem. **12:**31–39.
6. **Bennetzen, J. L., and B. D. Hall.** 1982. Codon selection in yeast. J. Biol. Chem. **257:**3026–3031.
7. **Bisson, L. F., D. M. Coons, A. L. Kruckeberg, and D. A. Lewis.** 1993. Yeast sugar transporters. CRC Crit. Rev. Biochem. Mol. Biol. **28:**259–308.
8. **Bonneaud, N., O. Ozier-Kalogeropoulos, G. Li, M. Labouesse, L. Minvielle-Sebastia, and F. Lacroute.** 1991. A family of low and high copy replicative, integrative and single-stranded *S. cerevisiae*/*E. coli* shuttle vectors. Yeast **7:**609–615.
9. **Borodovsky, M., K. E. Rudd, and E. V. Koonin.** 1994. Intrinsic and extrinsic approaches for detecting genes in a bacterial genome. Nucleic Acids Res. **22:**4756–4767.
10. **Bult, C. J., O. White, G. J. Olsen, L. Zhou, R. D. Fleischmann, et al.** 1996. Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. Science **273:**1058–1073.
11. **Coffman, J. A., R. Rai, T. S. Cunningham, V. Svetlov, and T. G. Cooper.** 1996. Gat1p, a GATA family protein whose production is sensitive to nitrogen catabolite repression, participates in transcriptional activation of nitrogen-catabolic genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:**847–858.
12. **Cooper, T. G.** 1981. Nitrogen metabolism and gene expression, p. 39–99. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces cerevisiae*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor Press, N.Y.
13. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
14. **Dubois, E., and M. Grenson.** 1979. Methylamine/ammonia uptake systems in *Saccharomyces cerevisiae*. Multiplicity and regulation. Mol. Gen. Genet. **175:** 67–76.
15. **Felsenstein, J.** 1989. PHYLIP—phylogeny inference package (version 3.2). Cladistics **5:**164–166.
16. **Frommer, W. B.** Personal communication.
17. **Grenson, M.** Unpublished data.
18. **Grenson, M.** 1973. Specificity and regulation of the uptake and retention of amino acids and pyrimidines in yeast, p. 179–193. *In* Z. Vanek, Z. Hostalek, and J. Culdin (ed.), Genetics of industrial microorganisms. Academia, Prague, Czechoslovakia.
19. **Grenson, M.** 1983. Inactivation reactivation process and repression of permease formation regulate several ammonia sensitive permeases in the yeast *Saccharomyces cerevisiae*. Eur. J. Biochem. **133:**135–139.
20. **Grenson, M.** 1992. Amino acid transporters in yeast: structure, function and regulation, p. 219–245. *In* J. J. L. L. M. De Pont (ed.), Molecular aspects of transport proteins. Elsevier Science, New York, N.Y.
21. **Grenson, M., M. Mousset, J.-M. Wiame, and J. Béchet.** 1966. Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae* I. Evidence for a specific arginine-transporting system. Biochim. Biophys. Acta **127:**325–338.
22. **Grenson, M., F. Muyldermans, K. Broman, and S. Vissers.** 1987. 4-Aminobutyric acid (GABA) uptake in baker's yeast *Saccharomyces cerevisiae* is mediated by the general amino acid permease, the proline permease and a GABA-specific permease integrated into the GABA-catabolic pathway. Life. Sci. Adv. Ser. C **6:**35–39.
23. **Hein, C., J.-Y. Springael, C. Volland, R. Haguenauer-Tsapis, and B. André.** 1995. NPI1, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the Rsp5 ubiquitin-protein ligase. Mol. Microbiol. **18:**77–87.
24. **Ito, H., Y. Fukuda, K. Muruta, and A. Kimura.** 1983. Transformation of intact yeast cells with alkali cations. J. Bacteriol. **153:**163–168.
25. **Jacobs, P., J.-C. Jauniaux, and M. Grenson.** 1980. A cis dominant regulatory mutation linked to the *argB-argC* gene cluster in *Saccharomyces cerevisiae*. J. Mol. Biol. **139:**691–704.
26. **Kaneko, T., S. Sato, H. Kotani, A. Tanaka, E. Asamizu, et al.** 1996. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis sp.* strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res. **3:**109–136.
27. **Ko, C. H., and R. F. Gaber.** 1993. Roles of multiple glucose transporters in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**638–648.
28. **Kyte, J., and R. F. Doolitle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157:**105–132.
29. **Lauter, F.-R., O. Ninnemann, M. Bucher, J. W. Riesmeier, and W. B. Frommer.** 1996. Preferential expression of an ammonium transporter and two putative nitrate transporters in root hairs of tomato. Proc. Natl. Acad. Sci. USA **93:**8139–8144.
30. **Lorenz, M., and J. Heitman.** 1996. Ammonia permeases are receptors for pseudohyphal growth, p. 3B. *In* Abstracts of the 1996 Yeast Genetics and Molecular Biology Meeting, Madison, Wis.
31. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
32. **Magasanik, B.** 1992. Regulation of nitrogen utilization. p. 283–317. *In* J. N. Strathern, E. W. Jones, and E. R. Broach (ed.), The molecular and cellular biology of the yeast *Saccharomyces*. II. Gene expression, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. **Mallet, L., F. Bussereau, and M. Jacquet.** 1995. A 43.5 kb segment of yeast chromosome XIV, which contains *MFA2*, *MEP2*, *CAP*/*SRV2*, *NAM9*, *FKB1*/*FPR1*/*RBP1*, *MOM22* and *CPT1*, predicts an adenosine deaminase gene and 14 new open reading frames. Yeast **11:**1195–1209.
34. **Marini, A.-M., S. Vissers, A. Urrestarazu, and B. André.** 1994. Cloning and expression of the *MEP1* gene encoding a transporter of ammonium in *Saccharomyces cerevisiae*. EMBO J. **13:**3456–3463.
35. **Ninnemann, O., J.-C. Jauniaux, and W. B. Frommer.** 1994. Identification of a high affinity ammonium transporter from plants. EMBO J. **13:**3464–3471.
36. **Özcan, S., J. Dover, A. G. Rosenwald, S. Wölfl, and M. Johnston.** 1996. Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. Proc. Natl. Acad. Sci. USA **93:**12428–12432.
37. **Özcan, S., and M. Johnston.** 1995. Three different regulatory mechanisms enable yeast hexose transporter (HXT) genes to be induced by different levels of glucose. Mol. Cell. Biol. **15:**1564–1572.
38. **Person, B., and P. Argos.** 1994. Prediction of transmembrane segments in proteins utilising multiple sequence alignments. J. Mol. Biol. **237:**182–192.
39. **Reifenberger, E., K. Freidel, and M. Ciriacy.** 1995. Identification of novel *HXT* genes in *Saccharomyces cerevisiae* reveals the impact of individual hexose transporters on glycolytic flux. Mol. Microbiol. **16:**157–167.
40. **Rost, B., and C. Sander.** 1993. Prediction of protein structure at better than 70% accuracy. J. Mol. Biol. **232:**584–599.
41. **Rothstein, R. J.** 1991. Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast, p. 281–301. *In* C. Guthrie and G. R. Fink (ed.), Guide to yeast genetics and molecular biology. Academic Press, Inc., New York, N.Y.
42. **Rupp, S., and G. R. Fink.** 1996. Mep2, a putative high affinity ammonium transporter, is required for filamentous growth, p. 119B. *In* Abstracts of the 1996 Yeast Genetics and Molecular Biology Meeting, Madison, Wis.
43. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
44. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring. Harbor Laboratory Press, Cold Spring Harbor, N.Y.
45. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
46. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Methods in yeast genetics.

Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
47. **Siewe, R. M., B. Weil, A. Burkovski, B. J. Eikmanns, M. Eikmanns, and R. Kramer.** 1996. Functional and genetic characterization of the (methyl)ammonium uptake carrier of *Corynebacterium glutamicum*. J. Biol. Chem. **271:** 5398–5403.
48. **Soussi-Boudekou, S., and B. André.** Unpublished data.
49. **Soussi-Boudekou, S., S. Vissers, A. Urrestarazu, J.-C. Jauniaux, and B. André.** 1997. Gzf3p, a fourth GATA factor involved in nitrogen-regulated transcription in *Saccharomyces cerevisiae*. Mol. Microbiol. **23:**1157–1168.
50. **Stanbrough, M., D. W. Rowen, and B. Magasanik.** 1995. Role of the GATA factors Gln3p and Nil1p of *Saccharomyces cerevisiae* in the expression of nitrogen-regulated genes. Proc. Natl. Acad. Sci. USA **92:**9450–9454.
51. **Tabor, C. W.** 1970. The determination of NH_3 with the use of glutamate dehydrogenase. Methods Enzymol. **17:**955.
52. **Wach, A., A. Brachat, R. Pohlmann, and P. Philippsen.** 1994. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast **10:**1793–1808.
53. **White, M. K., and M. J. Weber.** 1989. Leucine-zipper motif update. Nature **340:**103.
54. **Wiame, J.-M., M. Grenson, and H.N.J.R. Arst.** 1985. Nitrogen catabolite repression in yeasts and filamentous fungi. Adv. Microb. Physiol. **26:**1–87.
55. **Wilson, R., R. Ainscough, K. Anderson, C. Baynes, M. Berks, et al.** 1994. 2.2 MB of contiguous nucleotide sequence from chromosome III of *C. elegans*. Nature **368:**32–38.