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Role of the Npr1 Kinase in Ammonium Transport and Signaling by the Ammonium Permease Mep2 in *Candida albicans*▽

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The ammonium permease Mep2 induces a switch from unicellular yeast to filamentous growth in response to nitrogen limitation in *Saccharomyces cerevisiae* and *Candida albicans*. In *S. cerevisiae*, the function of Mep2 and other ammonium permeases depends on the protein kinase Npr1. Mutants lacking *NPR1* cannot grow on low concentrations of ammonium and do not filament under limiting nitrogen conditions. A G349C mutation in Mep2 renders the protein independent of Npr1 and results in increased ammonium transport and hyperfilamentous growth, suggesting that the signaling activity of Mep2 directly correlates with its ammonium transport activity. In this study, we investigated the role of Npr1 in ammonium transport and Mep2-mediated filamentation in *C. albicans*. We found that the two ammonium permeases Mep1 and Mep2 of *C. albicans* differ in their dependency on Npr1. While Mep1 could function well in the absence of the Npr1 kinase, ammonium transport by Mep2 was virtually abolished in *npr1*Δ mutants. However, the dependence of Mep2 activity on Npr1 was relieved at higher temperatures (37°C), and Mep2 could efficiently induce filamentous growth under limiting nitrogen conditions in *npr1*Δ mutants. Like in *S. cerevisiae*, mutation of the conserved glycine at position 343 in Mep2 of *C. albicans* to cysteine resulted in Npr1-independent ammonium uptake. In striking contrast, however, the mutation abolished the ability of Mep2 to induce filamentous growth both in the wild type and in *npr1*Δ mutants. Therefore, a mutation that improves ammonium transport by Mep2 under nonpermissible conditions eliminates its signaling activity in *C. albicans*.

Microorganisms sense the availability of nutrients in their environment and express appropriate transporters and enzymes that are required for uptake and metabolization of these nutrients (11, 16). A preferred nitrogen source for many microorganisms is ammonium, which is transported into the cell by ammonium permeases of the Mep/Amt family (31). The yeast *Saccharomyces cerevisiae* possesses three ammonium permeases encoded by the *MEP1* to *MEP3* genes. Each of these transporters can support growth of *S. cerevisiae* on media containing low concentrations of ammonium as the only nitrogen source, but mutants lacking all three *MEP* genes are unable to grow on ammonium at concentrations below 5 mM (21). Expression of the *MEP* genes is induced under limiting nitrogen conditions and repressed at high ammonium concentrations (21). Under the latter conditions, sufficient ammonium may freely diffuse into the cell in the form of ammonia or be taken up by unspecific transporters to support growth.

In addition to its transport function, Mep2 is required for the transition from yeast to filamentous, pseudohyphal growth, which occurs under limiting nitrogen conditions on solid media (13, 19). It is believed that Mep2 is an ammonium sensor that induces pseudohyphal growth in response to the presence of extracellular ammonium. Current evidence suggests that the signaling activity of Mep2 is linked to ammonium transport, because amino acid substitutions that inhibit the transport activity of Mep2 also prevent pseudohyphal growth (20, 26, 30).

Ammonium transport by Mep1 to Mep3 requires the serine/threonine protein kinase Npr1 (nitrogen permease reactivator 1), which is important for the activity of several permeases mediating uptake of nitrogenous compounds (9, 14, 15, 29). *S. cerevisiae npr1* mutants exhibit a growth defect on low-ammonium medium similar to that of mutants lacking the three ammonium permeases (10). Substitution of cysteine for the highly conserved glycine at position 349 in Mep2 results in a hyperactive transporter that is independent of Npr1 (4). The hyperactive Mep2 with the G349C mutation also induces filamentation more efficiently than does wild-type Mep2, further supporting a correlation between the ammonium transport and signaling activities of Mep2 (4).

The fungal pathogen *Candida albicans* also switches from budding yeast morphology to filamentous growth in response to different environmental signals, including nitrogen starvation (3). *C. albicans* has two ammonium permeases, Mep1 and Mep2, either of which is sufficient to enable growth in low ammonium concentrations (2). Similarly to the situation in *S. cerevisiae*, Mep2, but not Mep1, is required for filamentous growth of *C. albicans* in response to nitrogen limitation. The transport and signaling functions of Mep2 can be separated, as deletion of the C-terminal cytoplasmic tail of Mep2 abolishes filamentous growth without affecting ammonium uptake (2). Certain amino acid substitutions in Mep2 also disturb ammonium transport and/or filamentous growth (7). However, there is no direct correlation between the ammonium transport activity of mutated Mep2 proteins and their ability to stimulate filamentous growth. For example, mutation of the conserved residue W167 abolished filamentation without having a strong impact on ammonium transport. Vice versa, mutation of the conserved Y122, which is assumed to participate together with

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W167 in ammonium recruitment at the extracytosolic side of the cell membrane, reduced ammonium uptake more strongly than a W167A mutation but still allowed efficient filament formation (7). It is therefore possible that signaling by Mep2 is regulated in different ways in *C. albicans* and *S. cerevisiae*, and it has been proposed that a high transport activity of *C. albicans* Mep2 (CaMep2) in the presence of abundant extracellular ammonium may actually block its signaling activity and repress filamentous growth (2).

As the Npr1 kinase is essential for the function of Mep2 and the other ammonium permeases in *S. cerevisiae*, we investigated the role of Npr1 in ammonium uptake by Mep1 and Mep2 and in Mep2-mediated filamentous growth of *C. albicans*.

MATERIALS AND METHODS

Strains and growth conditions. *C. albicans* strains used in this study are listed in Table 1. All strains were stored as frozen stocks with 15% glycerol at −80°C and subcultured on YPD agar plates (20 g peptone, 10 g yeast extract, 20 g glucose, 20 g agar per liter) at 30°C. Strains were routinely grown in YPD liquid medium at 30°C in a shaking incubator. For selection of nourseothricin-resistant transformants, 200 μg/ml nourseothricin (Werner Bioagents, Jena, Germany) was added to YPD agar plates. To obtain nourseothricin-sensitive derivatives in which the *SAT1* flipper cassette was excised by FLP-mediated recombination, transformants were grown overnight in YPM medium (10 g yeast extract, 20 g peptone, 20 g maltose per liter) without selective pressure to induce the *MAL2* promoter, which controls expression of the *caFLP* gene ("*ca*" indicates *Candida-a*dapted gene) in the *SAT1* flipper cassette. One hundred to two hundred cells were spread on YPD plates containing 10 μg/ml nourseothricin and grown for 2 days at 30°C. Nourseothricin-sensitive clones were identified by their small colony size and confirmed by restreaking on YPD plates containing 100 μg/ml nourseothricin as described previously (25). For growth assays, YPD overnight cultures of the strains were washed two times in water and the cell suspensions adjusted to an optical density of 2. Ten microliters of a 10-fold dilution series (10^0 to 10^{-5}) was spotted on YPD and SD agar (1.7 g yeast nitrogen base without amino acids [YNB; BIO 101, Vista, CA], 20 g glucose, 15 g agar per liter) plates containing various concentrations of ammonium or other nitrogen sources (as indicated in the figures) and incubated at 30°C. Rapamycin sensitivity of the strains was tested on YPD agar plates containing 100 ng/ml rapamycin. Filamentation assays were performed by plating washed cells from a YPD overnight culture at a density of 30 to 50 cells per plate on SD 2% agar plates containing 100 μM ammonium or urea as described below. Colony phenotypes were recorded after 6 days of incubation at 37°C. Growth rates of the strains in SD medium containing 1 mM ammonium were determined at 30°C using a Bioscreen C analyzer (Growth Curves USA, Piscataway, NJ).

Plasmid constructions. Oligonucleotide primers used in this study are listed in Table 2. An *NPR1* deletion cassette was obtained by amplifying *NPR1* upstream and downstream sequences from genomic DNA of strain SC5314 by PCR with the primer pairs NPR1-1/NPR1-2 and NPR1-3/NPR1-4, respectively, and substituting the ApaI/XhoI- and SacII/SacI-digested PCR products for the *GAT1* flanking regions in the previously described plasmid pGAT1M2 (6) to generate pNPR1M2. For complementation of the *npr1*Δ mutants, a fragment containing the *NPR1* coding region and upstream sequences was amplified with the primers NPR1-1 and NPR1-5, digested with ApaI/BglII, and ligated together with a BglII-SalI fragment containing the *ACT1* transcription termination sequence (T_{ACT1}) from pMEP2K1 (2) into the ApaI/XhoI-digested pNPR1M2, yielding pNPR1K1. Plasmid pMEP1M5, which was used to delete the *MEP1* gene in prototrophic *C. albicans* strains, was generated by substituting the *SAT1* flipper cassette (25) for the *URA3* flipper cassette in the previously described pMEP1M2 (2). A deletion cassette for orf19.4446, which has similarity to ammonium permease genes and was designated *MEP3* for the purpose of the present study (although our results indicate that it does not encode a functional ammonium permease), was generated as follows. The orf19.4446 upstream and downstream sequences were amplified with the primer pairs MEP46/MEP47 and MEP48/MEP49, respectively, and the KpnI/XhoI- and BglII/SacI-digested PCR products substituted for the *MEP1* flanking sequences in pMEP1M2 to obtain pMEP3M2. The *URA3* flipper cassette was then replaced by the *SAT1* flipper cassette to generate pMEP3M3. To introduce a wild-type *MEP2* copy into *mep1*Δ *mep2*Δ double and *mep1*Δ *mep2*Δ *npr1*Δ triple mutants, a fragment containing the *MEP2* coding region and upstream sequences was amplified with the primers MEP3 and MEP79, digested with KpnI/BglII, and cloned together with a BglII-PstI T_{ACT1}-*caSAT1* fragment from pOPT1G22 (24) in the KpnI/PstI-digested pMEP2G6 (6), resulting in pMEP2K17. The $MEP2^{G343C}$ allele was obtained by an overlap PCR with the primer pairs MEP3/MEP108 and MEP107/ACT38 and substitution of the KpnI/BglII-digested PCR product for the corresponding fragment in pMEP2K17 to produce pMEP2K18. For reintroduction of *MEP1* into the *mep1*Δ *mep2*Δ double and *mep1*Δ *mep2*Δ *npr1*Δ triple mutants, the BglII-PstI T_{ACT1}-*caSAT1* fragment from pMEP2K17 was substituted for the corresponding fragment with the *URA3* marker in pMEP1K1 (2), generating pMEP1K3.

Strain constructions. *C. albicans* strains were transformed by electroporation (18) with the following gel-purified DNA fragments. The ApaI-SacI fragment from pNPR1M2 was used to delete *NPR1* in the wild-type strain SC5314 and the *mep1*Δ *mep2*Δ double mutants. The ApaI-SacI fragment from pNPR1K1 was used to reintegrate an intact *NPR1* copy into the *npr1*Δ mutants. The KpnI-SacI fragment from pMEP1M5 was used to delete *MEP1* in the *mep2*Δ mutants, and the KpnI-SacI fragment from pMEP3M3 was used to delete *MEP3* in the *mep1*Δ *mep2*Δ double mutants. The XhoI-SacI fragment from pMEP1K3 was used to reintegrate a wild-type *MEP1* copy into the *mep1*Δ *mep2*Δ double mutants and the *mep1*Δ *mep2*Δ *npr1*Δ triple mutants. The KpnI-SacI fragments from pMEP2K17 and pMEP2K18 were used to reintegrate wild-type *MEP2* and $MEP2^{G343C}$, respectively, into the *mep1*Δ *mep2*Δ double mutants and the *mep1*Δ *mep2*Δ *npr1*Δ triple mutants. The KpnI-SacI fragment from pMEP2G7 (6) was used to integrate a *GFP*-tagged *MEP2* copy into the *mep1*Δ *mep2*Δ double mutants and the *mep1*Δ *mep2*Δ *npr1*Δ triple mutants. The correct integration of all constructs was verified by Southern hybridization with gene-specific probes. During the course of this work, we noticed that one of the two independently constructed *mep1*Δ *mep2*Δ mutants and its derivatives (the B series) had become homozygous for chromosome R. However, in all assays described in this work, the two independently generated series of strains behaved identically, and the results obtained with these strains are therefore included.

Isolation of genomic DNA and Southern hybridization. Genomic DNA from *C. albicans* strains was isolated as described previously (25). DNA was digested with appropriate restriction enzymes, separated on a 1% agarose gel, and, after ethidium bromide staining, transferred by vacuum blotting onto a nylon membrane and fixed by UV cross-linking. Southern hybridization with enhanced-chemiluminescence-labeled probes was performed with an Amersham ECL direct nucleic acid labeling and detection system (GE Healthcare, Braunschweig, Germany) according to the instructions of the manufacturer.

Fluorescence microscopy. *C. albicans* strains expressing green fluorescent protein (GFP)-tagged Mep2 were grown overnight in SD medium containing 100 μM proline. Two milliliters of the overnight cultures was washed three times in water, resuspended in 10 ml SD medium containing 100 μM ammonium chloride, and incubated for 6 h at 30°C or 37°C. Fluorescence of the cells was detected using a Zeiss Observer Z1 microscope with a Zeiss HXP120C illuminator. Images were taken successively with filter settings for GFP and transmission images. Cells were observed with a 100× immersion oil objective.

Ammonium uptake assay. To determine ammonium uptake rates of the strains, an ammonium removal assay was used. Cells were grown overnight in 50 ml SD medium with 0.1% proline at 30°C, washed two times in water, and resuspended in SD medium with 1 mM ammonium chloride at an optical density of 2. The cultures were incubated with shaking (200 rpm) at 30°C in 30-ml volumes, and 1-ml samples were taken at 10 min, 30 min, 60 min, and then every 60 min until 6 h. The cells were pelleted, and 40 μl of the supernatant was added to 760 μl OPA solution (540 mg *o*-phthaldialdehyde, 10 ml ethanol, 50 μl β-mercaptoethanol, 0.2 M phosphate buffer, pH 7.3, at 100 ml) to quantify the remaining ammonium (1). After 20 min of incubation in the dark, the extinction at 420 nm was measured. As a reference, 760 μl OPA plus 40 μl water was used. The system was calibrated with ammonium chloride concentrations from 0 to 2 mM.

RESULTS

Requirement of the Npr1 kinase for growth of *C. albicans* on ammonium. To investigate whether the Npr1 kinase is required for ammonium uptake in *C. albicans*, we deleted the *NPR1* gene (orf19.6232) from the genome of the wild-type strain SC5314 by using the *SAT1*-flipping strategy (25). Two independent series of homozygous *npr1*Δ mutants and complemented strains (A and B series) were generated and tested for their ability to grow on agar plates containing

TABLE 1. *C. albicans* strains used in this study

Strain	Parent	Relevant characteristic or genotype[a]	Reference or source
SC5314		Wild-type parental strain	12
*npr1*Δ mutants and complemented strains			
NPR1M1A	SC5314	*npr1-1*::*SAT1-FLIP*/*NPR1-2*	This study
NPR1M1B	SC5314	*NPR1-1*/*npr1-2*::*SAT1-FLIP*	This study
NPR1M2A	NPR1M1A	*npr1-1*::*FRT*/*NPR1-2*	This study
NPR1M2B	NPR1M1B	*NPR1-1*/*npr1-2*::*FRT*	This study
NPR1M3A	NPR1M2A	*npr1-1*::*FRT*/*npr1-2*::*SAT1-FLIP*	This study
NPR1M3B	NPR1M2B	*npr1-1*::*SAT1-FLIP*/*npr1-2*::*FRT*	This study
NPR1M4A	NPR1M3A	*npr1-1*::*FRT*/*npr1-2*::*FRT*	This study
NPR1M4B	NPR1M3B	*npr1-1*::*FRT*/*npr1-2*::*FRT*	This study
NPR1MK1A	NPR1M4A	*NPR1-SAT1-FLIP*/*npr1-2*::*FRT*	This study
NPR1MK1B	NPR1M4B	*npr1-1*::*FRT*/*NPR1-SAT1-FLIP*	This study
NPR1MK2A	NPR1MK1A	*NPR1-FRT*/*npr1-2*::*FRT*	This study
NPR1MK2B	NPR1MK1B	*npr1-1*::*FRT*/*NPR1-FRT*	This study
*mep2*Δ mutants			
SCMEP2M4A	SC5314	*mep2-1*::*FRT*/*mep2-2*::*FRT*	6
SCMEP2M4B	SC5314	*mep2-1*::*FRT*/*mep2-2*::*FRT*	6
*mep1*Δ *mep2*Δ double mutants			
SCMEP12M1A	SCMEP2M4A	*MEP1-1*/*mep1-2*::*SAT1-FLIP* *mep2-1*::*FRT*/*mep2-2*::*FRT*	This study
SCMEP12M1B	SCMEP2M4B	*mep1-1*::*SAT1-FLIP*/*MEP1-2* *mep2-1*::*FRT*/*mep2-2*::*FRT*	This study
SCMEP12M2A	SCMEP12M1A	*MEP1-1*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT*	This study
SCMEP12M2B	SCMEP12M1B	*mep1-1*::*FRT*/*MEP1-2* *mep2-1*::*FRT*/*mep2-2*::*FRT*	This study
SCMEP12M3A	SCMEP12M2A	*mep1-1*::*SAT1-FLIP*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT*	This study
SCMEP12M3B	SCMEP12M2B	*mep1-1*::*FRT*/*mep1-2*::*SAT1-FLIP* *mep2-1*::*FRT*/*mep2-2*::*FRT*	This study
SCMEP12M4A	SCMEP12M3A	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT*	This study
SCMEP12M4B	SCMEP12M3B	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT*	This study
*mep1*Δ *mep2*Δ *mep3*Δ triple mutants			
SCMEP123M1A	SCMEP12M4A	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT* *mep3-1*::*SAT1-FLIP*/*MEP3-2*	This study
SCMEP123M1B	SCMEP12M4B	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT* *MEP3-1*/*mep3-2*::*SAT1-FLIP*	This study
SCMEP123M2A	SCMEP123M1A	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT* *mep3-1*::*FRT*/*MEP3-2*	This study
SCMEP123M2B	SCMEP123M1B	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT* *MEP3-1*/*mep3-2*::*FRT*	This study
SCMEP123M3A	SCMEP123M2A	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT* *mep3-1*::*FRT*/*mep3-2*::*SAT1-FLIP*	This study
SCMEP123M3B	SCMEP123M2B	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT* *mep3-1*::*SAT1-FLIP*/*mep3-2*::*FRT*	This study
SCMEP123M4A	SCMEP123M3A	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT* *mep3-1*::*FRT*/*mep3-2*::*FRT*	This study
SCMEP123M4B	SCMEP123M3B	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT* *mep3-1*::*FRT*/*mep3-2*::*FRT*	This study
*mep1*Δ *mep2*Δ *npr1*Δ triple mutants			
Δ*mep12*NPR1M1A	SCMEP12M4A	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT* *npr1-1*::*SAT1-FLIP*/*NPR1-2*	This study
Δ*mep12*NPR1M1B	SCMEP12M4B	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT* *NPR1-1*/*npr1-2*::*SAT1-FLIP*	This study
Δ*mep12*NPR1M2A	Δ*mep12*NPR1M1A	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT* *npr1-1*::*FRT*/*NPR1-2*	This study

Continued on following page

TABLE 1—*Continued*

Strain	Parent	Relevant characteristic or genotype[a]	Reference or source
Δ*mep12*NPR1M2B	Δ*mep12*NPR1M1B	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT* *NPR1-1*/*npr1-2*::*FRT*	This study
Δ*mep12*NPR1M3A	Δ*mep12*NPR1M2A	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT* *npr1-1*::*FRT*/*npr1-2*::*SAT1-FLIP*	This study
Δ*mep12*NPR1M3B	Δ*mep12*NPR1M2B	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT* *npr1-1*::*SAT1-FLIP*/*npr1-2*::*FRT*	This study
Δ*mep12*NPR1M4A	Δ*mep12*NPR1M3A	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT* *npr1-1*::*FRT*/*npr1-2*::*FRT*	This study
Δ*mep12*NPR1M4B	Δ*mep12*NPR1M3B	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT* *npr1-1*::*FRT*/*npr1-2*::*FRT*	This study
Strains expressing *MEP1*, *MEP2*, or *MEP2*^{G343C} in *mep1*Δ *mep2*Δ or *mep1*Δ *mep2*Δ *npr1*Δ backgrounds			
SCΔ*mep12*MEP1K1A	SCMEP12M4A	*mep1-1*::*FRT*/*MEP1-caSAT1* *mep2-1*::*FRT*/*mep2-2*::*FRT*	This study
SCΔ*mep12*MEP1K1B	SCMEP12M4A	*MEP1-caSAT1*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT*	This study
SCΔ*mep12*MEP2K1A	SCMEP12M4A	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*MEP2-caSAT1*	This study
SCΔ*mep12*MEP2K1B	SCMEP12M4B	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*MEP2-caSAT1*	This study
SCΔ*mep12*MEP2K2A	SCMEP12M4A	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*MEP2*^{G343C}*-caSAT1*	This study
SCΔ*mep12*MEP2K2B	SCMEP12M4B	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*MEP2*^{G343C}*-caSAT1*	This study
Δ*mep1*Δ*mep2*Δ*npr1*MEP1K1A	Δ*mep12*NPR1M4A	*mep1-1*::*FRT*/*MEP1-caSAT1* *mep2-1*::*FRT*/*mep2-2*::*FRT* *npr1-1*::*FRT*/*npr1-2*::*FRT*	This study
Δ*mep1*Δ*mep2*Δ*npr1*MEP1K1B	Δ*mep12*NPR1M4B	*MEP1-caSAT1*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*mep2-2*::*FRT* *npr1-1*::*FRT*/*npr1-2*::*FRT*	This study
Δ*mep1*Δ*mep2*Δ*npr1*MEP2K1A	Δ*mep12*NPR1M4A	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*MEP2-caSAT1* *npr1-1*::*FRT*/*npr1-2*::*FRT*	This study
Δ*mep1*Δ*mep2*Δ*npr1*MEP2K1B	Δ*mep12*NPR1M4B	*mep1-1*::*FRT*/*mep1-2*::*FRT* *MEP2-caSAT1*/*mep2-2*::*FRT* *npr1-1*::*FRT*/*npr1-2*::*FRT*	This study
Δ*mep1*Δ*mep2*Δ*npr1*MEP2K2A	Δ*mep12*NPR1M4A	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*MEP2*^{G343C}*-caSAT1* *npr1-1*::*FRT*/*npr1-2*::*FRT*	This study
Δ*mep1*Δ*mep2*Δ*npr1*MEP2K2B	Δ*mep12*NPR1M4B	*mep1-1*::*FRT*/*mep1-2*::*FRT* *MEP2*^{G343C}*-caSAT1*/*mep2-2*::*FRT* *npr1-1*::*FRT*/*npr1-2*::*FRT*	This study
Strains expressing *GFP*-tagged *MEP2* in *mep1*Δ *mep2*Δ or *mep1*Δ *mep2*Δ *npr1*Δ backgrounds			
SCΔ*mep12*MEP2G7A	SCMEP12M4A	*mep1-1*::*FRT*/*mep1-2*::*FRT* *MEP2-GFP-caSAT1*/*mep2-2*::*FRT*	This study
SCΔ*mep12*MEP2G7B	SCMEP12M4B	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*MEP2-GFP-caSAT1*	This study
SCΔ*mep12*Δ*npr1*MEP2G7A	Δ*mep12*NPR1M4A	*mep1-1*::*FRT*/*mep1-2*::*FRT* *MEP2-GFP-caSAT1*/*mep2-2*::*FRT* *npr1-1*::*FRT*/*npr1-2*::*FRT*	This study
SCΔ*mep12*Δ*npr1*MEP2G7B	Δ*mep12*NPR1M4B	*mep1-1*::*FRT*/*mep1-2*::*FRT* *mep2-1*::*FRT*/*MEP2-GFP-caSAT1* *npr1-1*::*FRT*/*npr1-2*::*FRT*	This study

[a] *SAT1-FLIP* denotes the *SAT1* flipper cassette. The *NPR1*, *MEP1*, *MEP2*, and *MEP3* alleles in strain SC5314 can be distinguished by HaeII, ClaI, EcoRI, and BamHI restriction site polymorphisms, respectively. The *NPR1* allele containing the polymorphic HaeII site at position +1002 was arbitrarily designated *NPR1-2*, the *MEP1* allele with the polymorphic upstream ClaI site *MEP1-1*, the *MEP2* allele with the polymorphic upstream EcoRI site *MEP2-1*, and the *MEP3* allele with the polymorphic downstream BamHI site *MEP3-2*.

different concentrations of ammonium as the only nitrogen source. As can be seen in Fig. 1A, the *npr1*Δ mutants had a slight growth defect on these media. However, the growth defect was not as severe as that of mutants lacking the ammonium permeases Mep1 and Mep2, indicating that ammonium can still be taken up into the cells in the absence of Npr1, albeit not as efficiently as in the wild-type strain. A reduced growth rate of the *npr1*Δ mutants was also observed in liquid medium containing 1 mM ammonium (Table 3). Ammonium uptake assays demonstrated that ammonium uptake by the *npr1*Δ mutants was reduced to ca. 30% of wild-type uptake rates (Fig. 1B).

TABLE 2. Primers used in this study

Primer name	Primer sequence[a]
ACT38	5′-ATATGGGCCCTGCAGACATTTTATGATGGAATGAATGGG-3′
NPR1-1	5′-ATATGGGCCCTTCCAAGGCACGAGACC-3′
NPR1-2	5′-ATATCTCGAGGCAGTTGACTTTGCTGCAGTG-3′
NPR1-3	5′-ATATCCGCGGTGGGAATCATTACGAAGATC-3′
NPR1-4	5′-ATATGAGCTCCCGTTGGTGAAGTGTCGTTG-3′
NPR1-5	5′-ATATAGAT**CTA**TTTCTTCTTATTCTTTTCAAAAGC-3′
MEP3	5′-TAAATACGGTACCCAAACGATTGGCTTGAATGTC-3′
MEP46	5′-TACCACTGGTACCCGATTATCTAATTAC-3′
MEP47	5′-TCAACATCTCGAGACATGGCTGACGG-3′
MEP48	5′-ATAATACAGATCTTACATAGTACGATAC-3′
MEP49	5′-CACTTATGAGCTCTTGATTGCCAGAG-3′
MEP79	5′-AAAGAACAGAT**CTA**ATTTTTAGCTTCTCC-3′
MEP107	5′-GTGTGGGCACTCCAT*TGT*GTTGGTGG-3′
MEP108	5′-CCACCAAC*ACA*ATGGAGTGCCCACAC-3′

[a] Restriction sites introduced into the primers are underlined; stop codons (reverse sequence) are highlighted in bold and mutated codons in italics.

Differential requirement of Npr1 for ammonium transport by Mep1 and Mep2. The phenotype of the *npr1*Δ mutants suggested that, in contrast to the situation in *S. cerevisiae*, one or both of the *C. albicans* ammonium permeases Mep1 and Mep2 can function in a partially Npr1-independent fashion. To address the specific requirement of Npr1 for Mep1 and/or Mep2 activity, we deleted *NPR1* in a *mep1*Δ *mep2*Δ double mutant background and then reintroduced a functional copy of *MEP1* or *MEP2* into the double and triple mutants. Reinsertion of *MEP1* into the *mep1*Δ *mep2*Δ double mutants restored growth and ammonium uptake to wild-type levels (Fig. 2 and Table 3). *MEP2* also complemented the growth defect of the

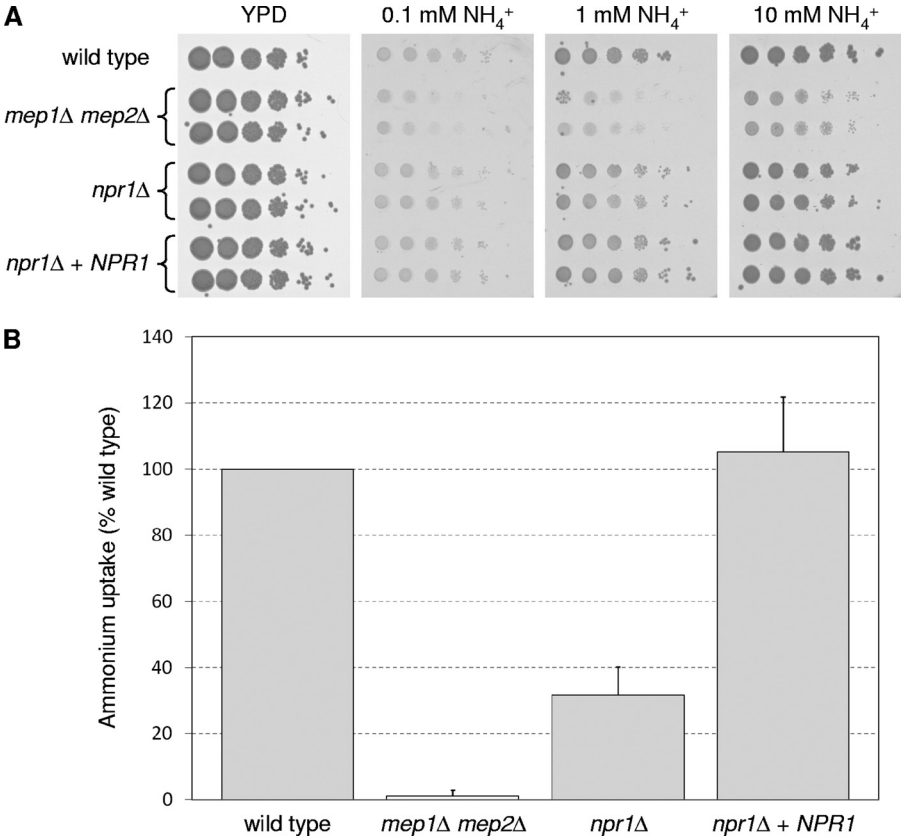

FIG. 1. (A) Growth of the wild-type strain SC5314, *mep1*Δ *mep2*Δ double mutants, and *npr1*Δ mutants and complemented strains on different concentrations of ammonium as the sole nitrogen source. Tenfold dilution series of the strains were spotted on a YPD control plate or on SD plates containing the indicated ammonium concentrations and incubated for 1 day (YPD) or 3 days (SD) at 30°C. The following strains were used: SC5314 (wild type), SCMEP12M4A and -B (*mep1*Δ *mep2*Δ), NPR1M4A and -B (*npr1*Δ), and NPR1MK2A and -B (*npr1*Δ + *NPR1*). (B) Ammonium uptake by the same strains. Uptake rates were determined in the presence of 1 mM ammonium as described in Materials and Methods. Ammonium uptake by the wild-type strain SC5314 was set to 100%, and uptake rates by the mutants are given as percentages of the wild-type uptake rate. Shown are the means and standard deviations from three independent experiments (one with the A series and two with the B series of mutants).

TABLE 3. Doubling times of *C. albicans* strains in liquid medium with 1 mM NH_4^+

Strain	Description or genotype	Doubling time (min)[a]
SC5314	Wild type	111
NPR1M4	*npr1*Δ	229 ± 14
NPR1MK2	*npr1*Δ + *NPR1*	125 ± 12
SCMEP12M4	*mep1*Δ *mep2*Δ	1,215 ± 70
SCΔ*mep12*MEP1K1	*mep1*Δ *mep2*Δ + *MEP1*	113 ± 5
SCΔ*mep12*MEP2K1	*mep1*Δ *mep2*Δ + *MEP2*	145 ± 27
SCΔ*mep12*MEP2K2	*mep1*Δ *mep2*Δ + *MEP2*^{G343C}	170 ± 17
Δ*mep12*NPR1M4	*mep1*Δ *mep2*Δ *npr1*Δ	1,305 ± 154
Δ*mep1*Δ*mep2*Δ*npr1*MEP1K1	*mep1*Δ *mep2*Δ *npr1*Δ + *MEP1*	266 ± 21
Δ*mep1*Δ*mep2*Δ*npr1*MEP2K1	*mep1*Δ *mep2*Δ *npr1*Δ + *MEP2*	1,056 ± 64
Δ*mep1*Δ*mep2*Δ*npr1*MEP2K2	*mep1*Δ *mep2*Δ *npr1*Δ + *MEP2*^{G343C}	193 ± 4

[a] Means ± standard deviations (SD) from four biological replicates (two each for the A and B series). The doubling time of strain SC5314 is the means from two biological replicates (106 min and 116 min).

*mep1*Δ *mep2*Δ double mutants on agar plates containing limiting ammonium concentrations (Fig. 2A), but ammonium uptake was still reduced (Fig. 2B), in agreement with a slightly slower growth of the strains in liquid medium (Table 3).

A more striking difference between *MEP1* and *MEP2* was seen in strains lacking the Npr1 kinase. Reintegration of *MEP1* into the *mep1*Δ *mep2*Δ *npr1*Δ triple mutants strongly improved growth both on solid and in liquid medium, although ammonium uptake was only partially restored. In contrast, expression of *MEP2* in the same strains only slightly ameliorated ammonium uptake and growth (Fig. 2 and Table 3), indicating that Mep2 does not function efficiently in the absence of Npr1.

To evaluate whether expression or localization of Mep2 was impaired in the absence of Npr1, we expressed a GFP-tagged

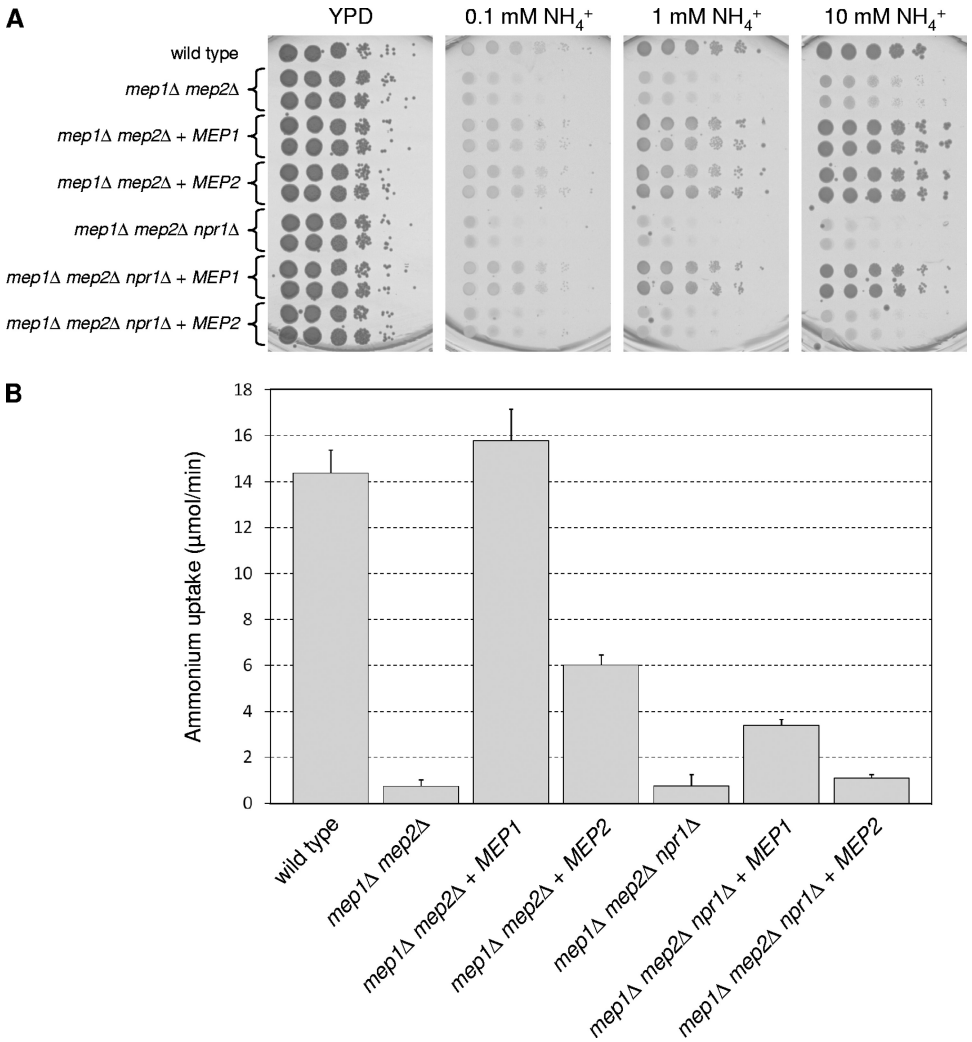

FIG. 2. (A) Growth of the wild-type strain SC5314, *mep1*Δ *mep2*Δ double mutants, *mep1*Δ *mep2*Δ *npr1*Δ triple mutants, and strains in which a functional copy of *MEP1* or *MEP2* was reinserted on different concentrations of ammonium as the sole nitrogen source. Tenfold dilution series of the strains were spotted on a YPD control plate or on SD plates containing the indicated ammonium concentrations and incubated for 1 day (YPD) or 3 days (SD) at 30°C. The following strains were used: SC5314 (wild type), SCMEP12M4A and -B (*mep1*Δ *mep2*Δ), SCΔ*mep12*MEP1K1A and -B (*mep1*Δ *mep2*Δ + *MEP1*), SCΔ*mep12*MEP2K1A and -B (*mep1*Δ *mep2*Δ + *MEP2*), Δ*mep12*NPR1M4A and -B (*mep1*Δ *mep2*Δ *npr1*Δ), Δ*mep1*Δ*mep2*Δ*npr1*MEP1K1A and -B (*mep1*Δ *mep2*Δ *npr1*Δ + *MEP1*), and Δ*mep1*Δ*mep2*Δ*npr1*MEP2K1A and -B (*mep1*Δ *mep2*Δ *npr1*Δ + *MEP2*). (B) Ammonium uptake by the same strains. Uptake rates were determined in the presence of 1 mM ammonium as described in Materials and Methods. Shown are the means and standard deviations from three independent experiments (two with the A series and one with the B series of mutants).

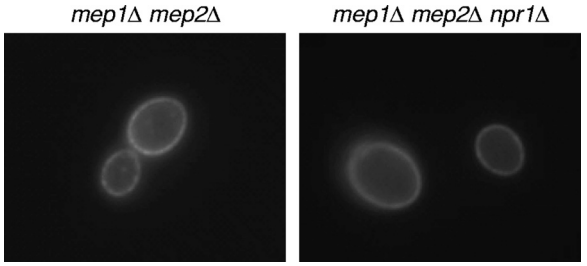

FIG. 3. Expression of GFP-tagged Mep2 in *mep1*Δ *mep2*Δ double mutants (strains SCΔ*mep12*MEP2G7A and -B) and *mep1*Δ *mep2*Δ *npr1*Δ triple mutants (strains SCΔ*mep12*Δ*npr1*MEP2G7A and -B). Cells were grown in SD medium containing 100 μM ammonium at 30°C and observed by fluorescence microscopy. The two independently generated series of mutants behaved identically, and only one of them is shown in each case.

Mep2 in the same strains. As can be seen in Fig. 3, Mep2 was localized at the cell periphery and expressed at similar levels in the presence or absence of Npr1, demonstrating that Npr1 is not required for expression and correct localization of Mep2. Altogether, these results argue that ammonium transport by Mep2 strongly depends on Npr1, while Mep1 can efficiently support growth in the absence of the kinase.

Npr1 is required for ammonium uptake by unspecific transporters. Of note, deletion of *NPR1* in a *mep1*Δ *mep2*Δ double mutant background resulted in a further growth impairment (Fig. 2A and Table 3), although no difference between *mep1*Δ *mep2*Δ double mutants and *mep1*Δ *mep2*Δ *npr1*Δ triple mutants could be detected in ammonium uptake assays (Fig. 2B). These results indicated that Npr1 is required for the activity of other proteins that contribute to ammonium uptake/utilization. The most obvious candidate for an Npr1-dependent ammonium transporter was orf19.4446, which encodes a putative protein with 34% amino acid identity to Mep1 and Mep2. To test whether this protein, which we designated Mep3 for the purpose of the present study, is responsible for the residual ammonium uptake in the absence of Mep1 and Mep2, we deleted the corresponding gene in a *mep1*Δ *mep2*Δ double mutant background. However, no growth differences between *mep1*Δ *mep2*Δ double mutants and *mep1*Δ *mep2*Δ *mep3*Δ triple mutants were observed at a range of ammonium concentrations (Fig. 4), indicating that orf19.4446 does not encode a functional ammonium permease. It is likely that Npr1 is required for the activity of unspecific cation transporters that allow some ammonium uptake into the cells in the absence of the specific ammonium transporters Mep1 and Mep2, especially at increased ammonium concentrations.

Npr1 is required for growth of *C. albicans* on other nitrogen sources. In *S. cerevisiae*, Npr1 is required for growth on other nitrogen sources in addition to ammonium, in part because the ammonium permeases contribute to growth under these conditions by retrieval of excreted ammonium (4). We therefore tested growth of the *C. albicans npr1*Δ mutants on a panel of these alternative nitrogen sources (Fig. 5). Similarly to *S. cerevisiae npr1*Δ mutants, *C. albicans* mutants lacking *NPR1* exhibited a growth defect on isoleucine, tyrosine, and tryptophan. However, there were also notable differences in the phenotypes of *npr1*Δ mutants of the two species. While Npr1 has been shown to be required for growth of *S. cerevisiae* on arginine, ornithine, and urea, little or no growth defect of the *C. albicans npr1*Δ mutants was observed on these nitrogen sources. Vice versa, inactivation of *NPR1* in *C. albicans* severely affected the ability of the mutants to utilize threonine and phenylalanine, whereas Npr1 is not required for growth of *S. cerevisiae* on these amino acids. In *C. albicans*, the ammonium permeases Mep1 and Mep2 contributed only marginally to growth on most of the tested alternative nitrogen sources, except for ornithine and isoleucine, where a relatively strong growth defect of the *mep1*Δ *mep2*Δ mutants was observed. These results suggest that the activity of certain amino acid transporters or enzymes involved in the metabolization of the corresponding amino acids depends on Npr1. However, the exacerbated growth defect of the *mep1*Δ *mep2*Δ *npr1*Δ triple mutants compared to that of the *npr1*Δ single mutants demonstrates that ammonium retrieval by Mep1 is important for the residual growth of *npr1*Δ mutants on various amino acids.

Mep2-mediated ammonium transport and filamentous growth become independent of Npr1 at elevated temperatures. In both *S. cerevisiae* and *C. albicans*, Mep2 also acts as a signaling protein and stimulates filamentous growth in response to nitrogen limitation. As explained in the introduction, the ability of *S. cerevisiae* Mep2 (ScMep2) to induce filamentation correlates with its ammonium transport activity. Consequently, *S. cerevisiae npr1*Δ mutants have a filamentation defect, because Npr1 is required for Mep2 activity (4, 19). As our previous experiments demonstrated that Npr1 is important for ammonium transport by Mep2 also in *C. albicans*, we investigated whether the *npr1*Δ mutants had a filamentation defect under limiting nitrogen conditions. Filamentous growth is usu-

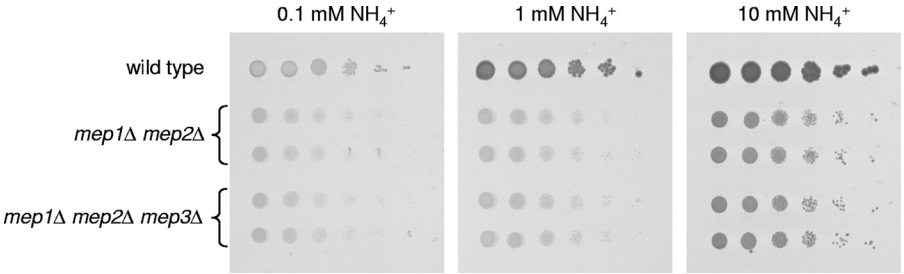

FIG. 4. Growth of the wild-type strain SC5314, *mep1*Δ *mep2*Δ double mutants, and *mep1*Δ *mep2*Δ *mep3*Δ triple mutants on different concentrations of ammonium as the sole nitrogen source. Tenfold dilution series of the strains were spotted on SD plates containing the indicated ammonium concentrations and incubated for 3 days at 30°C. The following strains were used: SC5314 (wild type), SCMEP12M4A and -B (*mep1*Δ *mep2*Δ), and SCMEP123M4A and -B (*mep1*Δ *mep2*Δ *mep3*Δ).

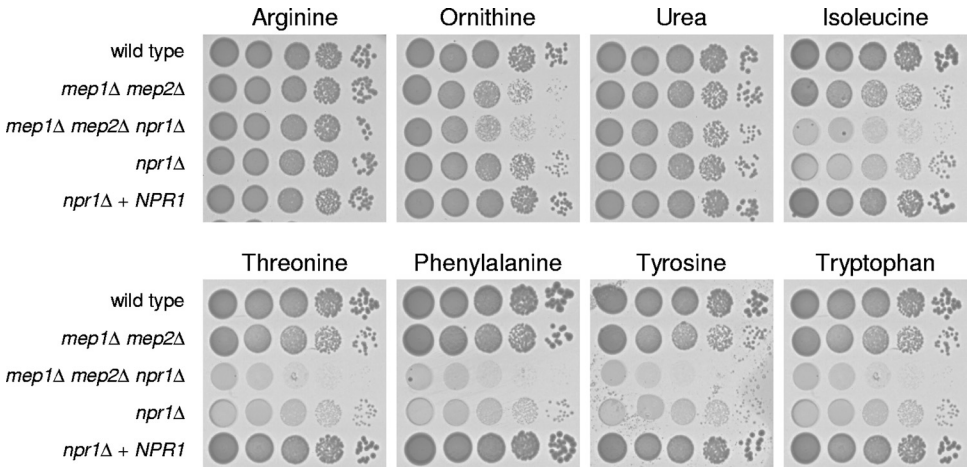

FIG. 5. Growth of the wild-type strain SC5314 and different mutants on SD agar plates containing the indicated amino acids (10 mM) as the sole nitrogen source. Tenfold dilution series of the strains were spotted on the plates and incubated for 3 days at 30°C. The following strains were used: SC5314 (wild type), SCMEP12M4A and -B (*mep1*Δ *mep2*Δ), Δ*mep12*NPR1M4A and -B (*mep1*Δ *mep2*Δ *npr1*Δ), NPR1M4A and -B (*npr1*Δ), and NPR1MK2A and -B (*npr1*Δ + *NPR1*). The two independently generated series of mutants behaved identically, and only one of them is shown in each case.

ally stimulated at elevated temperatures in *C. albicans*. Surprisingly, we observed that Npr1 is much less important for Mep2-dependent growth at 37°C than at 30°C, as growth of *mep1*Δ *mep2*Δ *npr1*Δ triple mutants expressing a single copy of *MEP2* was largely restored at the elevated temperature (Fig. 6A). Analysis of strains expressing a GFP-tagged Mep2 showed that Mep2 was expressed at comparable levels at 30°C and 37°C, both in the absence and in the presence of Npr1 (data not shown). In line with the Npr1-independent activity of Mep2 at 37°C, the *C. albicans npr1*Δ mutants exhibited fila-

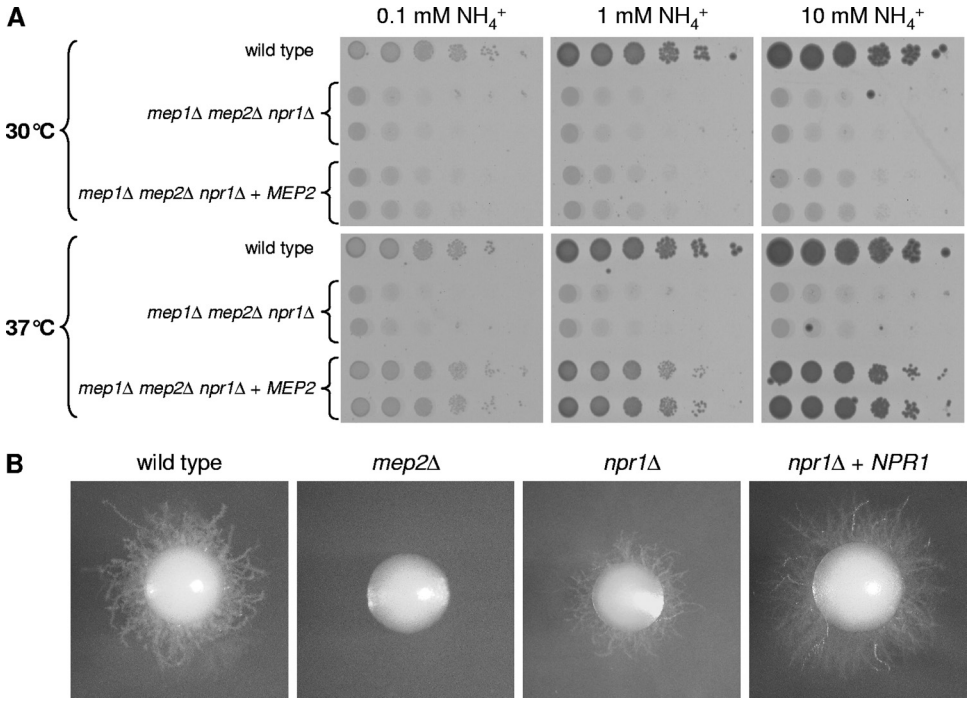

FIG. 6. (A) Mep2-dependent growth becomes independent of the Npr1 kinase at elevated temperatures. Tenfold dilution series of the strains were spotted on SD plates containing the indicated ammonium concentrations and incubated for 3 days at 30°C or 37°C. The following strains were used: SC5314 (wild type), Δ*mep12*NPR1M4A and -B (*mep1*Δ *mep2*Δ *npr1*Δ), and Δ*mep1*Δ*mep2*Δ*npr1*MEP2K1A and -B (*mep1*Δ *mep2*Δ *npr1*Δ + *MEP2*). (B) Filamentous growth of the wild-type strain SC5314, *mep2*Δ mutants, and *npr1*Δ mutants and complemented strains on SD agar plates containing 100 μM ammonium as the sole nitrogen source. The plates were incubated for 6 days at 37°C. The following strains were used: SC5314 (wild type), SCMEP2M4A and -B (*mep2*Δ), NPR1M4A and -B (*npr1*Δ), and NPR1MK2A and -B (*npr1*Δ + *NPR1*). The two independently generated series of mutants behaved identically, and only one of them is shown in each case.

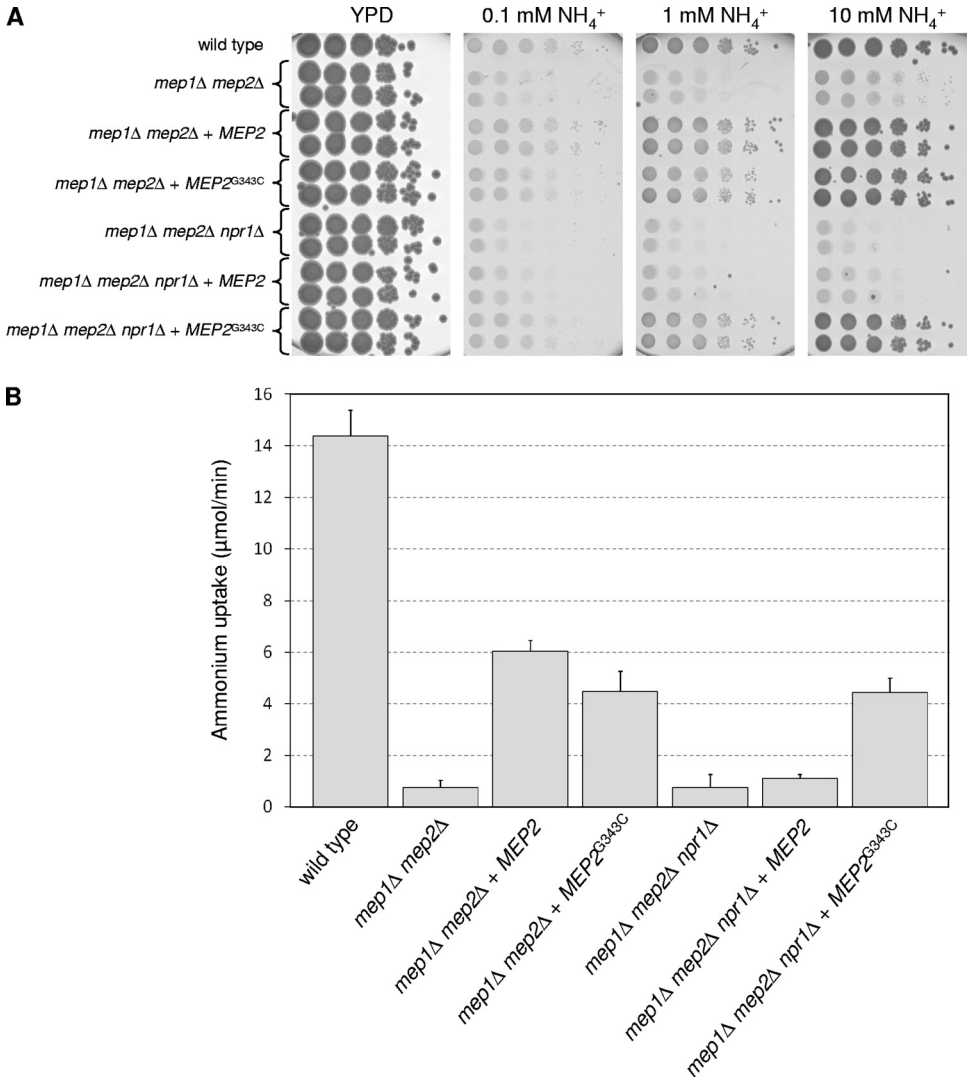

FIG. 7. (A) Growth of the wild-type strain SC5314, *mep1*Δ *mep2*Δ double mutants, *mep1*Δ *mep2*Δ *npr1*Δ triple mutants, and strains in which a wild-type copy of *MEP2* or the *MEP2*^{G343C} allele was reinserted on different concentrations of ammonium as the sole nitrogen source. Tenfold dilution series of the strains were spotted on a YPD control plate or on SD plates containing the indicated ammonium concentrations and incubated for 1 day (YPD) or 3 days (SD) at 30°C. The following strains were used: SC5314 (wild type), SCMEP12M4A and -B (*mep1*Δ *mep2*Δ), SCΔ*mep12*MEP2K1A and -B (*mep1*Δ *mep2*Δ + *MEP2*), SCΔ*mep12*MEP2K2A and -B (*mep1*Δ *mep2*Δ + *MEP2*^{G343C}), Δ*mep12*NPR1M4A and -B (*mep1*Δ *mep2*Δ *npr1*Δ), Δ*mep1*Δ*mep2*Δ*npr1*MEP2K1A and -B (*mep1*Δ *mep2*Δ *npr1*Δ + *MEP2*), and Δ*mep1*Δ*mep2*Δ*npr1*MEP2K2A and -B (*mep1*Δ *mep2*Δ *npr1*Δ + *MEP2*^{G343C}). (B) Ammonium uptake by the same strains. Uptake rates were determined in the presence of 1 mM ammonium as described in Materials and Methods. Shown are the means and standard deviations from three independent experiments (two with the A series and one with the B series of mutants). Data are from the same experiments with results shown in Fig. 2, and the values of the control strains are included for comparison.

mentous growth on media containing limiting ammonium concentrations, although filamentation was slightly reduced in the absence of Npr1 (Fig. 6B).

A mutation that restores Mep2-mediated ammonium transport in the absence of Npr1 abolishes the signaling activity of Mep2. As a G349C mutation in ScMep2 results in a hyperactive, Npr1-independent ammonium transporter, we investigated the effect of a corresponding G343C mutation in CaMep2. Similarly to the situation in *S. cerevisiae*, the *MEP2*^{G343C} allele restored growth of *C. albicans mep1*Δ *mep2*Δ *npr1*Δ triple mutants on media containing ammonium as the sole nitrogen source (Fig. 7A and Table 3), and ammonium uptake rates by the mutated Mep2 were similar in the presence and absence of Npr1 (Fig. 7B). Therefore, the G343C mutation resulted in Npr1-independent ammonium transport activity of Mep2 also in *C. albicans*. When expressed in a *mep1*Δ *mep2*Δ mutant, *MEP2*^{G343C} restored ammonium uptake and growth slightly less efficiently than did wild-type *MEP2*.

We then tested the ability of the wild-type *MEP2* and *MEP2*^{G343C} alleles to complement the filamentation defect of *mep1*Δ *mep2*Δ mutants in the presence and absence of Npr1. Unexpectedly, the *MEP2*^{G343C} allele was unable to induce filamentous growth under limiting nitrogen conditions, in contrast to wild-type *MEP2*, which induced morphogenesis regard-

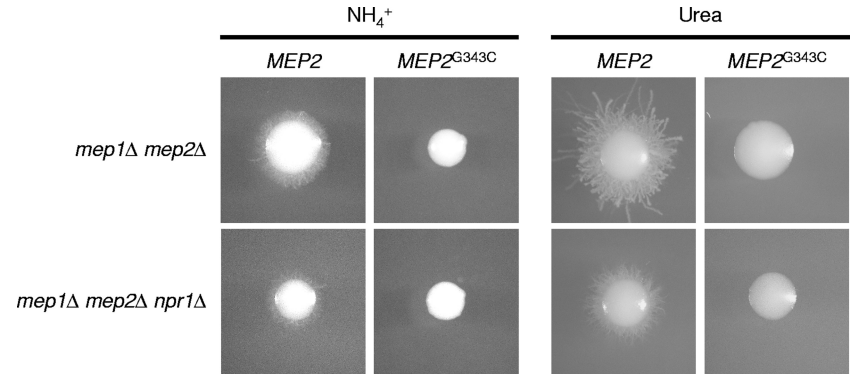

FIG. 8. Filamentous growth of *mep1*Δ *mep2*Δ double mutants and *mep1*Δ *mep2*Δ *npr1*Δ triple mutants expressing wild-type *MEP2* or the *MEP2*^{G343C} allele on SD agar plates containing 100 μM ammonium or urea as the sole nitrogen source. The plates were incubated for 6 days at 37°C. The following strains were used: SCΔ*mep12*MEP2K1A and -B (*mep1*Δ *mep2*Δ + *MEP2*), SCΔ*mep12*MEP2K2A and -B (*mep1*Δ *mep2*Δ + *MEP2*^{G343C}), Δ*mep1*Δ*mep2*Δ*npr1*MEP2K1A and -B (*mep1*Δ *mep2*Δ *npr1*Δ + *MEP2*), and Δ*mep1*Δ*mep2*Δ*npr1*MEP2K2A and -B (*mep1*Δ *mep2*Δ *npr1*Δ + *MEP2*^{G343C}). The two independently generated series of mutants behaved identically, and only one of them is shown in each case.

less of the presence of Npr1 (Fig. 8). Therefore, a mutation in Mep2 that restores the ammonium transport capacity in the absence of the Npr1 kinase abolishes the ability to induce filamentous growth.

DISCUSSION

The results presented in this work demonstrate that the importance of the Npr1 kinase for ammonium permease function in *S. cerevisiae* is different from that in *C. albicans*. The transport activity of all three ammonium permeases of *S. cerevisiae* strongly depends on Npr1, and none of the Mep proteins can support growth at low ammonium concentrations in the absence of this kinase (4, 10). In contrast, the two ammonium permeases of *C. albicans* differ in their dependency on Npr1. While Mep1 was sufficiently active in cells lacking Npr1 to support growth under low-ammonium conditions, ammonium transport by Mep2 was largely abolished in *npr1*Δ mutants at the standard growth temperature of 30°C. Interestingly, however, at 37°C Mep2 functioned well in the absence of Npr1 and enabled growth on ammonium as the sole nitrogen source.

In *S. cerevisiae*, Npr1 is required to maintain the general amino acid permease Gap1, which is expressed when the cells are grown in a nitrogen-poor medium, at the cytoplasmic membrane by preventing ubiquitination-dependent endocytosis. In the absence of Npr1, Gap1 is endocytosed and targeted to the vacuole for degradation, and newly synthesized Gap1 is directly sorted to the vacuole and never reaches the plasma membrane (8, 28). There is evidence that Npr1 does not phosphorylate Gap1 directly but acts in an indirect fashion by phosphorylating the α-arrestin Aly2, thereby interfering with Gap1 sorting to the vacuole (23). On the other hand, the nitrate transporter Ynt1 of *Hansenula polymorpha* is protected from ubiquitinylation-mediated sorting to the vacuole by direct, Npr1-dependent phosphorylation in response to nitrogen deprivation, allowing its delivery to the plasma membrane (22). Npr1 itself is inhibited under nutrient-rich conditions by the TOR (target of rapamycin) kinase signaling pathway and activated upon nitrogen limitation by Sit4-dependent dephosphorylation (17, 27). Inactivation of *NPR1* confers increased resistance to rapamycin in *S. cerevisiae* (27), and we observed the same phenotype for the *C. albicans npr1*Δ mutants (data not shown), indicating that Npr1 activity is controlled by TOR also in *C. albicans*.

We found that the role of Npr1 in maintaining the transport activity of Mep2 in *C. albicans* is different from its role in stabilizing Gap1 in *S. cerevisiae*, as Mep2 was properly expressed and localized in *C. albicans npr1*Δ mutants at the restrictive temperature of 30°C. Similar results have been reported for *S. cerevisiae*, where Mep2 was also correctly targeted to the plasma membrane in the absence of Npr1 (26). Therefore, instead of preventing endocytosis and degradation of Mep2, Npr1 seems to enable Mep2 to adopt a transport-competent conformation. Apparently, CaMep2 can attain its active conformation at higher temperatures also in an Npr1-independent fashion. Additional observations suggest that the role of Npr1 in maintaining ammonium permease function in *S. cerevisiae* is not protection from ubiquitination-dependent degradation. Gap1 degradation in the absence of Npr1 is prevented when ubiquitination is inhibited by an *rsp5* mutation (8). In contrast, nitrogen catabolite repression (NCR)-sensitive genes were still expressed at normally repressing ammonium concentrations in an *npr1* mutant even when *RSP5* was also inactivated, indicating that ammonium permease activity in the *npr1* mutant was not restored by the *rsp5* mutation (10). These findings support the idea that the role of Npr1 in maintaining Mep function is different from its role in stabilizing Gap1 at the plasma membrane.

Our results indicate that ammonium permeases can attain a transport-competent state by different mechanisms in *C. albicans*. Mep2 becomes transport proficient in the presence of a functional Npr1 kinase, at elevated temperatures, or by a G343C mutation. Interestingly, Mep1 also contains the conserved glycine, mutation of which to cysteine in Mep2 of *S. cerevisiae* and *C. albicans* renders these transporters Npr1 independent. Therefore, the ability of CaMep1 to efficiently transport ammonium in the absence of Npr1 must be caused by some other feature of this permease in which it differs from Mep2.

In general, the signaling activity of mutated Mep2 proteins

in *S. cerevisiae* correlates with their transport activity, supporting the model that ammonium transport is required for signaling by Mep2 (4, 20, 26, 30). On the other hand, an H194E mutation abolished pseudohyphal growth despite the fact that it increased ammonium transport by Mep2 (5). Therefore, the possibility that the signaling activity of Mep2 is in fact repressed when it is engaged in ammonium transport, especially in the presence of relatively high ammonium concentrations, cannot be ruled out. So far, no mutations in Mep2 that prevent ammonium transport and result in constitutive signaling, which would be in favor of such an alternative model for the regulation of Mep2 signaling activity, have been described. Nevertheless, our finding that one mechanism by which Mep2 achieves a transport-competent state, namely, the G343C mutation, abolishes its signaling activity is compatible with this hypothesis. However, the ammonium uptake rate of Mep2 was slightly reduced by the G343C mutation in a wild-type background, which may be the reason for the filamentation defect. In *S. cerevisiae*, the analogous G349S mutation has a different effect in that it increases both the transport and signaling activities of Mep2 above those of the wild-type protein. It is therefore possible that the signaling activity of Mep2 is controlled in different ways by ammonium availability in the two yeast species.

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