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Impact of Ammonium Permeases MepA, MepB, and MepC on Nitrogen-Regulated Secondary Metabolism in *Fusarium fujikuroi*[▽]

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In *Fusarium fujikuroi*, the production of gibberellins and bikaverin is repressed by nitrogen sources such as glutamine or ammonium. Sensing and uptake of ammonium by specific permeases play key roles in nitrogen metabolism. Here, we describe the cloning of three ammonium permease genes, *mepA*, *mepB*, and *mepC*, and their participation in ammonium uptake and signal transduction in *F. fujikuroi*. The expression of all three genes is strictly regulated by the nitrogen regulator AreA. Severe growth defects of Δ*mepB* mutants on low-ammonium medium and methylamine uptake studies suggest that MepB functions as the main ammonium permease in *F. fujikuroi*. In Δ*mepB* mutants, nitrogen-regulated genes such as the gibberellin and bikaverin biosynthetic genes are derepressed in spite of high extracellular ammonium concentrations. *mepA mepB* and *mepC mepB* double mutants show a similar phenotype as Δ*mepB* mutants. All three *F. fujikuroi mep* genes fully complemented the *Saccharomyces cerevisiae mep1 mep2 mep3* triple mutant to restore growth on low-ammonium medium, whereas only MepA and MepC restored pseudohyphal growth in the *mep2/mep2* mutant. Overexpression of *mepC* in the Δ*mepB* mutants partially suppressed the growth defect but did not prevent derepression of AreA-regulated genes. These studies provide evidence that MepB functions as a regulatory element in a nitrogen sensing system in *F. fujikuroi* yet does not provide the sensor activity of Mep2 in yeast, indicating differences in the mechanisms by which nitrogen is sensed in *S. cerevisiae* and *F. fujikuroi*.

The sensing and uptake of nitrogen are essential processes for fungal growth and development. Ammonium and glutamine are preferred nitrogen sources (48), as their presence results in the repression of genes that are involved in the acquisition and utilization of other sources of nitrogen. This regulatory system is known as nitrogen metabolite repression in filamentous fungi (53) or nitrogen catabolite repression (NCR) in *Saccharomyces cerevisiae* (6). A family of GATA-type transcription factors that activate gene expression when levels of preferred nitrogen sources become limiting mediates this regulation. Members of this family include AreA from *Aspergillus nidulans* and *Fusarium fujikuroi*, NIT2 from *Neurospora crassa*, and Gln3p from *S. cerevisiae* (12, 21, 26, 50).

Under nitrogen starvation conditions, the rice pathogenic fungus *F. fujikuroi* produces the red pigment bikaverin and gibberellins (GAs), mainly the gibberellic acids GA_3, GA_4, and GA_7, the causative agents of the "bakanae" disease of rice seedlings. GAs are isoprenoid plant hormones used as plant growth regulators in agriculture and horticulture (40). Bikaverin is a polyketide which is responsible for the deep red color exhibited under certain growth conditions by cultures of some *Fusarium* species (23). Interestingly, neither of these secondary metabolites contains nitrogen, and they do not have any obvious function in nitrogen metabolism (reviewed in reference 51). Both the GA and bikaverin biosynthetic genes are strictly repressed by nitrogen. For the GA biosynthetic genes a direct dependence on AreA has already been shown (33, 46, 47, 50), whereas the role of AreA in regulation of bikaverin genes is not yet clear.

Additional components of nitrogen metabolism also influence the correct expression of the GA and bikaverin biosynthetic genes in *F. fujikuroi*. Glutamine synthetase (GS) is required for the wild-type expression of a variety of genes, including those involved in ribosome biogenesis and translation initiation, which are induced in a mutant strain lacking GS (46). Surprisingly, GS activity is also required for the expression of the GA and bikaverin biosynthetic genes, which is abolished, rather than induced, in a GS null mutant (46). This suggests an important role for GS in control of cellular metabolism and highlights the complexity of the regulatory networks that respond to nitrogen metabolism in *F. fujikuroi*.

The GA and bikaverin biosynthetic genes are also targets of the TOR signaling pathway (47). In yeast, the conserved TOR (target of rapamycin) kinases play a significant role in nutrient sensing and cell growth by affecting nuclear localization of transcription factors, such as Gln3, and thereby controlling genes subject to NCR (reviewed in reference 41). In *F. fujikuroi*, several AreA-regulated genes, e.g., GA and bikaverin biosynthetic genes, are only partially derepressed by rapamycin suggesting that additional regulatory pathways involved in nitrogen metabolism exist.

We are interested in identifying components that mediate nitrogen sensing and which act upstream of the transcription

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factor AreA in *F. fujikuroi*. In yeast and filamentous fungi, nitrogen permeases, such as ammonium and amino acid permeases, can form part of the nitrogen regulatory network (15). Ammonium transport in fungi is mediated by permeases that belong to the conserved AmtB/Mep family of proteins that are related to the mammalian rhesus blood group antigens. These proteins are highly conserved within bacteria, plants, and animals and share a similar protein structure (13, 16, 18, 29, 31, 49). Within certain fungal species, one of the permeases has evolved a regulatory function. Examples include Mep2 (*S. cerevisiae* and *Candida albicans*), Amt1 (*Hebeloma cylindrosporum*), and Ump2 (*Ustilago maydis*), which are required for the induction of filamentous growth under low-nitrogen conditions (3, 18, 24, 25, 44, 52). In *S. cerevisiae*, this dimorphic transition requires the cooperation of two signaling pathways, the mitogen-activated protein kinase and the cyclic AMP-dependent pathways (22).

It is not clear at present the extent to which regulatory ammonium permeases are conserved within fungal species. In this study we have characterized three members of the AmtB/Mep family from *F. fujikuroi* that we have designated MepA, MepB, and MepC. Phenotypic and methylamine uptake studies in *S. cerevisiae* confirm that they are functional ammonium permeases. The genes encoding these permeases are under AreA-mediated nitrogen metabolite repression control. MepA and MepC but not MepB fully restored pseudohyphal growth in the *S. cerevisiae mep2* mutant. MepB is probably the major ammonium permease in *F. fujikuroi*, and its deletion results in derepression of the GA and bikaverin biosynthetic genes and other genes subject to nitrogen metabolite repression under ammonium-sufficient conditions. Furthermore, overexpression of *mepC* in the Δ*mepB* background partially suppresses the strong growth defect but not the derepression of AreA target genes on high ammonium concentrations. We suggest, therefore, a sensing or regulatory role of MepB in addition to its function as a permease.

MATERIALS AND METHODS

Fungal strains and culture conditions. Strain IMI58289 (Commonwealth Mycological Institute, Kew, United Kingdom) is a GA-producing wild-type strain of *F. fujikuroi*. For regulation studies, the *areA* deletion strain T19 (50), the *gdhA* deletion mutant (B. Tudzynski, unpublished data), and the *glnA* mutant (46) were used. For all cultivations, *F. fujikuroi* strains were precultivated for 48 h in 300-ml Erlenmeyer flasks with 100 ml of Darken medium (DVK) (8) with 2.0 g/liter glutamine instead of $(NH_4)_2SO_4$ on a rotary shaker; 1 ml of this culture was used as inoculum for cultivations in ICI (Imperial Chemical Industries Ltd., United Kingdom) medium.

For DNA isolation and protoplasting, *F. fujikuroi* strains were incubated in complete medium (39) at 28°C on a rotary shaker at 200 rpm for 3 days or 18 h, respectively. For analysis of GA production, the fungus was grown for 5 days at 28°C on a rotary shaker (190 rpm) in a liquid production medium containing 60 g/liter sunflower oil, 15 g/liter corn steep solids (Sigma, Germany), 1.0 g/liter glutamine, and 1.0 g/liter KH_2PO_4 or in ICI medium (14) with 20% of the ammonium nitrate concentration (20% ICI). The *S. cerevisiae* strains MLY40α (*ura3-52 MAT*α), MLY131α (*mep1*::*LEU2 mep2*::*LEU2 mep3*::*G418 ura3-52 MAT*α), MLY97**a**/α (*ura3-52*/*ura3-52 leu2*::*hisG*/*leu2*::*hisG MAT***a**/α), and MLY108**a**/α (*mep2*::*LEU2*/*mep2*::*LEU2 ura3-52*/*ura3-52 leu2*::*hisG*/*leu2*::*hisG MAT***a**/α) were used to analyze the function of the *F. fujikuroi mep* genes (24, 25).

Bacterial strains and plasmids. *Escherichia coli* strain Top10 (Invitrogen, Groningen, The Netherlands) was used for plasmid propagation. Genomic DNA fragments carrying the *F. fujikuroi mepA*, *mepB*, and *mepC* genes or parts of the genes were cloned into the vector pUC19 (Fermentas, Germany). For the replacement of *mepA*, a 1.1-kb KpnI/SalI fragment of the 5′ noncoding region and a 0.9-kb HindIII/BamHI fragment of the 3′ noncoding region were cloned into the plasmid pUCH2-8 (1) carrying the hygromycin B resistance cassette. A KpnI/BamHI fragment of the resulting replacement vector, pΔmepA, carrying both flanks and the hygromycin resistance cassette, was used for gene replacement experiments. To construct the *mepB* gene replacement vector, a 0.8-kb SacII/XbaI fragment of the 5′ noncoding region and a 0.5-kb ClaI/SalI fragment from the 3′ noncoding region were cloned into the plasmid pNRI (28) carrying the nourseothricin resistance gene *nat1*. For targeted replacement of *mepC*, a 0.7-kb SacI/XbaI fragment of the 5′ noncoding region and a 0.8-kb HindIII/SalI right flank were cloned into vector pNR1.

For complementation of the *mepB* mutant with the wild-type *mepB* copy, a 4.8-kb genomic SacI fragment was cloned into the vector pPUCH2-8 (1) carrying the hygromycin resistance cassette. The circular vector pmepB-hyg was used to transform the *mepB* mutant strain T1 (*mepB*-T1, where T1 is for transformant 1). For constructing the *mepC* overexpression vector, the promoter of the *F. fujikuroi glnA* gene (46) was amplified by PCR using the primers glnA-prom-XbaI and glnA-prom-Bam. The coding region of the *mepC* gene was amplified with the primers MepC-Bam-F and MepC-Hind-R. The restricted promoter and *mepC* fragments were cloned by one step into the XbaI/HindIII-restricted vector pUCH2-8.

Screening of genomic library. About 40,000 recombinant phages of the *F. fujikuroi* m567 genomic library (28) were plated with *E. coli* strain XII-Blue MRF′ and screened by plaque hybridization as described previously (42). Plaque lifts (Gene Screen nylon membranes; DuPont, Germany) were hybridized with [^{32}P]dCTP-labeled 0.5-kb PCR fragments of the *F. fujikuroi mepA*, *mepB*, and *mepC* genes. Hybridization and washing steps were performed at 65°C. The blots were washed at 65°C (once with 2 × SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.1% sodium dodecyl sulfate [SDS]; once with 0.1 × SSC and 0.1% SDS) as described previously (46). Putative positive phages were purified in a second screening round. Phage DNA was isolated as described previously (42) and used for restriction analysis and subcloning.

DNA isolation. Lyophilized mycelium was ground into a fine powder with a mortar and pestle and dispersed (in the case of DNA for use in PCR) in extraction buffer as described by Cenis (5). DNA for Southern hybridization experiments was prepared following the protocol of Doyle and Doyle (9). Plasmid DNA was extracted using a plasmid extraction kit (Genomed, Germany).

Southern-blot analysis. For Southern blot analysis, genomic, plasmid, or phage DNA was digested to completion with appropriate restriction enzymes (Fermentas, Germany), fractionated in 1.0% (wt/vol) agarose gels, and transferred to nylon N$^+$ membranes (Amersham, Germany) by vacuum blotting. DNA probes were randomly labeled, and hybridizations were carried out overnight at 65°C. The blots were washed under hybridization conditions (2 × SSC–0.1% SDS at 65°C followed by 0.1 × SSC–0.1% SDS).

Northern blot analysis. For RNA isolation, the fungal strains were grown in ICI medium with 20 mM glutamine as a nitrogen source instead of ammonium nitrate for 3 days on a rotary shaker at 28°C. After 24, 48, and 72 h, mycelia were harvested and used for RNA preparation. For shift experiments, the mycelium was grown for 3 days in ICI medium with 20 mM glutamine, washed, and transferred into synthetic ICI medium without nitrogen for 2 h to induce starvation; the mycelium was then shifted into medium without nitrogen or with 10 mM NH_4NO_3 or 10 mM glutamine, with (200 ng/ml) or without rapamycin (Calbiochem). Mycelia were harvested after 1 h. The irreversible inhibitor of the GS, L-methionine sulfoximine (MSX) (Sigma-Aldrich, Germany), was added in a concentration of 4.0 mM. Total *F. fujikuroi* RNA was isolated using an RNAgents total RNA isolation kit (Promega, Germany).

PCR and reverse transcription-PCR (RT-PCR). PCR primers MepL 5′-CAA TGGTTCTTCTGGGGCTACTC-3′ and MepR 5′-CGAACCAGCCGA ACCA GAGGAA-3′ were used for amplification of *F. fujikuroi* ammonium permease genes *mepA* and *mepB*. For cloning the third ammonium permease gene, *mepC*, primers MepC-F1 5′-CTTCAACATGTCTTATGTTATCCC-3′ and MepC-R1 5′- GCAAGTTTAAGCATGCTTCTCG-3′ were used. To construct gene replacement vectors pΔmepA, pΔmepB, and pΔmepC, the 3′ and 5′ noncoding regions were amplified with the following primers: epA-GR1-KpnI, 5′-GGAAT TCGGTACCCTTGAGGAC-3′; MepA-GR2-SalI, 5′-TGTAAGGAGTGGTCA TGTCGAGCAGACTGC-3′; MepA-GR3-Hind, 5′-TAAGCTTCGAGAAGAA GTGGAGCACC-3′; MepA-GR4-BamHI, 5′-CGGATCCTTGATAGGAGTA TAAAGGACC-3′; MepB-GR1-SacII, 5′-CGAAAAAAACGTCACCGCGGTA CCGG-3′; MepB-GR2-XbaI, 5′-CTAGAGGAACCATTAACAGCACTAGAG C-3′; MepB-GR3-ClaI, 5′-GATCGATCGATATCGATAACGAGTCCG-3′; MepB-GR4-SalI, 5′-CGTCGACTTCAGACTGCTGCTATTAGTCC-3′; MepC-GR1-SacI, 5′-TTGGGGAGCTCGTACTGATAAACATCCATGAGATGG-3′; MepC-GR2-XbaI, 5′-GCAATAAAATAACCCCTCTAGATCAAACAAGG-3′; MepC-GR3-HindIII, 5′-GGTGATTAACGAGAAGCATGCTTAAGCTTG

C-3′; MepC-GR4-SalI, 5′-ATGTCGACGTTACTCTGGCTCCCAGATCAAC ACG-3′.

Derived fragments were first cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced. For constructing the yeast expression vectors, the full-length cDNA fragments of the three *F. fujikuroi mep* genes were generated by RT-PCR using the following primers: MepA-Sca, 5′-GCAGTACTGTAAAAATGACCA CTCC-3′; MepA-Xba, 5′-CACTAATCTAGACCTTTAGACC-3′; MepB-Sca, 5′-GCAGCCTAGTACTATCGCAAAAATGCTCTCG-3′; MepB-Xba, 5′-GGT ATCTAGAACTCGACAATTCC-3′; MepC-ScaI, 5′-CCACGAGTACTTATA AACACAATAATCAAAAATGTC-3′; and MepC-Xba, 5′-GCAATAAAATA ACCCCTCTAGATCAAACAAGG-3′.

The cDNA fragments of *mepA*, *mepB*, and *mepC* were first cloned into the PCR cloning vector pCR2.1-TOPO (Invitrogen) and then subcloned into the ScaI/XbaI-restricted yeast expression vector pYES2.1 (Invitrogen) to yield complementation vectors pYes2.1-mepA, pYes2.1-mepB, and pYes2.1-mepC. These plasmids were sequenced and used for complementation of the yeast triple *mep1 mep2 mep3* and *mep2* mutants. Vector pYes2.1-mepB was also used for complementing the *F. fujikuroi* Δ*mepB* mutant.

For constructing the *mepC* overexpression vector, the following primers were used: MepC-Bam-F, 5′-ATGGATCCAACATGTCTTATGTTATCCCTGG-3′; MepC-Hind-R, 5′-ATAAGCTTCTTTGCGTGCTACTCCTAGCATCCCGC-3′; glnA-prom-Xba, 5′-AGTCTAGACGGAGCAAAGCGGTTTATATCCGC C-3′; and glnA-prom-Bam, 5′-TTGGATCCTGTGAATGTGGTTGTGATACG GGG-3′.

PCRs contained 25 ng of DNA, 50 ng of each primer, a 0.2 mM concentration of each deoxynucleoside triphosphate, and 2 U of *Taq* polymerase (Red Taq; Sigma-Aldrich, Germany) in 50 μl. PCR was carried out at 94°C for 4 min followed by 30 cycles of 94°C for 1 min, 53 to 57°C for 30 s, and 72°C for 1.5 min. For RT-PCR 1 μg of total RNA of nitrogen-starved wild-type mycelium served as a template to create cDNA by using a One-step qRT-PCR kit (Invitrogen, Groningen, Germany).

Fungal transformations. Preparation of protoplasts from *F. fujikuroi* mycelium was carried out as described previously (50). A total of 10^7 protoplasts of strain IMI58289 were transformed with 10 μg of the KpnI/BamHI, SacI/SalI, or SacI/SalI fragment of the replacement vectors pΔmepA, pΔmepB, or pΔmepC, respectively. For gene replacement, transformed protoplasts were regenerated at 28°C in a complete regeneration agar (0.7 M sucrose, 0.05% yeast extract, 0.1% Casamino Acids) containing 120 μg/ml hygromycin B (for pΔmepA) (Calbiochem, Germany) or 100 μg/ml nourseothricin (for pΔmepB and pΔmepC) (Werner-Bioagents, Germany) for 6 to 7 days. For construction of *mepA mepB* double mutants, strain Δ*mepA*-T10 was transformed with 10 μg of the SacI/SalI-fragment of vector pΔmepB. For construction of *mepB mepC* double mutants, strain Δ*mepB*-T1 was cotransformed with 10 μg of the SacI/SalI fragment of vector pΔmepC and 10 μg of the vector pUCH2-8 carrying the hygromycin resistance cassette. For complementation, the Δ*mepB*-T1 mutant was cotransformed with 10 μg (each) of the hygromycin B resistance-mediating vector pUCH2-8 and the pYES2.1 vector carrying the *mepB* cDNA fragment. The transformants were additionally selected by regeneration on a medium containing low ammonium concentrations (1 mM ammonium citrate) as the only nitrogen source. Single conidial cultures were established from hygromycin B- or nourseothrecin-resistant transformants and used for DNA isolation and Southern blot analysis.

Analysis of *mepA*, *mepB*, and *mepC* function in *S. cerevisiae*. To test the function of the *F. fujikuroi mep* genes in *S. cerevisiae*, the vectors pYes2.1-mepA, pYes2.1-mepB, and pYes-mepC and control vectors pYes2.1-Mep2 and pYes2.1 were transformed into the haploid *S. cerevisiae* strain MLY131α (*mep1 mep2 mep3*). The wild-type MLY40α containing pYes2.1 served as a control strain. Growth of transformants under ammonium-limiting conditions was assayed by plating serial dilutions of washed overnight cultures onto SD plates (yeast nitrogen base with ammonium sulfate and without amino acid supplements plus 2% glucose) and SLADG plates (yeast nitrogen base without ammonium sulfate and without amino acids supplemented with 50 μM ammonium sulfate, 2% galactose, and 0.2% glucose). Pseudohyphal growth was analyzed by streaking transformants of the diploid MLY108a/α (*mep2*/*mep2*) *S. cerevisiae* strain containing the *F. fujikuroi* pYes2.1-mep vectors to single cells onto SLADG agar, which were then grown for 6 days at 30°C and photographed.

DNA sequencing and sequence homology searches. DNA sequencing of recombinant plasmid clones was accomplished with the automatic sequencer Li-Cor 4000 (MWG, München, Germany). The two strands of overlapping subclones obtained from the genomic DNA clones were sequenced using the universal and the reverse primers or specific IRD-800-labeled oligonucleotides obtained from MWG Biotech (Munich, Germany). DNA and protein sequence alignments were done with DNA Star (Madison, WI). Sequence homology searches were performed using the NCBI database server. Protein homology was based on BlastX searches (2). For further investigations, the programs of DNA STAR Inc. (Madison, WI) were used.

[^{14}C]methylamine uptake. The analysis of methylamine uptake was carried out as previously described (44). Briefly, the *S. cerevisiae* diploid *mep1 mep2 mep3* null strain was transformed with the yeast vector pYES2.1 that contained *mepA*, *mepB*, and *mepC* under the control of the *GAL1* promoter. Individual transformants were grown in synthetic medium lacking uracil with galactose (3%) as the carbon source and proline as the nitrogen source (1 mM) for 6 h. The cells were pelleted, washed, and resuspended in phosphate buffer (20 mM; pH 7) to an optical density at 595 nm of 8 and incubated on ice. Aliquots were added to phosphate buffer (20 mM; pH 7) containing 0.1 mCi [^{14}C]methylamine hydrochloride (MP Biomedicals, Inc) and increasing concentrations of methylamine. The cells were resuspended in a water bath at 30°C, and samples of 1 ml were removed at 1-min intervals and washed over GC Whatman filters. The level of [^{14}C]methylamine was then quantified using liquid scintillation counting.

GA determination by TLC. Amounts of produced GA3 and GA4/7 were determined by thin-layer chromatography (TLC) on silica gel eluted with ethyl acetate-chloroform-acetic acid (60:40:5).

Nucleotide sequence accession numbers. The sequences of the *F. fujikuroi mepA*, *mepB*, and *mepC* genes were deposited in the GenBank database under accession numbers AM168272, AM168273, and AM283470, respectively.

RESULTS

Cloning of the permease genes *mepA*, *mepB*, and *mepC*. Primers based on the conserved MEP sequences from other fungi were used to isolate homologues from *F. fujikuroi*. Isolated PCR fragments were used to probe a genomic library and resulted in the isolation of two ammonium permease genes, *mepA* and *mepB*. During the course of this work, we noted that the genome of *Fusarium verticillioides*, a close relative of *F. fujikuroi*, contains a third *mep* gene. Primers based on the sequence of this gene were used to amplify the coding region and regulatory sequences of a third ammonium permease-encoding gene, *mepC* in *F. fujikuroi*. The three genes are predicted to encode proteins of 466 (MepA), 502 (MepB), and 513 (MepC) amino acids. All three permeases share a high degree of sequence similarity with other permeases of the AmtB/Mep family (Table 1). A phylogenetic tree of protein sequences of known ammonium transporters of the Mep/Amt family revealed a clear cluster of fungal ammonium permeases that is distinct from those of animals, plants, and bacteria (Fig. 1). *F. fujikuroi* MepC is most similar to MeaA from *A. nidulans* (fungal family II), and both MepA and MepB group together with MepA from *A. nidulans* in the main group of fungal ammonium permeases. The three *F. fujikuroi* permeases are predicted to contain 11 transmembrane helices with an N_{out}-C_{in} topology, based on the TMHMM and HMMTOP prediction programs (45), and this prediction is in agreement with predictions for other fungal ammonium transporters (32, 49) (Fig. 2). In addition, putative glycosylation sites are predicted for MepA (N6), MepB (N16), and MepC (N13) by the program PROSITE (43).

Analysis of *mepA*, *mepB*, and *mepC* gene expression. The three *F. fujikuroi mep* genes are repressed when cells are grown in medium containing nitrogen sources such as ammonium, glutamine, arginine, and nitrate at concentrations of 10 and 100 mM, whereas weak expression of the *mep* genes is observed when 10 mM glutamate is used as a nitrogen source (data not shown). To determine if *mep* gene expression is dependent on AreA, we compared the expression pattern of the *mep* genes in wild-type cells and in the *areA* and *glnA* mutants. Both strains were initially grown for 5 days in a

TABLE 1. Sequence similarities of the *F. fujikuroi* Mep proteins with other permeases of the AmtB/Mep family

F. fujikuroi Mep	AmtB/Mep family protein (accession no.)	Description	Organism[a]	% Sequence identity
MepA	FG02094		*F. graminearum*	91
	MepA (CAD21326)		*N. crassa*	64
	MepA (AAL73118)	High affinity	*A. nidulans*	62
	AMT1 (AAL11032)	High affinity	*T. borchii*	63
	Ump2 (XM756943)	High affinity; sensor	*U. maydis*	56
	MeaA (AAL73117)	High capacity	*A. nidulans*	52
	Mep2 (P41948)	High affinity, low capacity; sensor	*S. cerevisiae*	50
MepB	FG00620		*F. graminearum*	97
	AMT1 (AAL11032)	High affinity	*T. borchii*	77
	MepA (AAL73118)	High affinity	*A. nidulans*	79
	MepA (CAD21326)		*N. crassa*	71
	MepC		*F. fujikuroi*	64
	Ump2 (XM756943)	High affinity; sensor	*U. maydis*	59
	Mep2 (P41948)	High affinity, low capacity, sensor	*S. cerevisiae*	50
MepC	FG00529		*F. graminearum*	94
	MeaA (AAL73117)	High capacity	*A. nidulans*	73
	MepA (AAL73118)	High affinity	*A. nidulans*	58
	Mep1 (CAA97132)	High capacity, low affinity	*S. cerevisiae*	57
	Mep3 (P53390)	High capacity, low affinity	*S. cerevisiae*	56
	AMT1 (AAL11032)	High affinity	*T. borchii*	56
	MepA	High affinity	*F. fujikuroi*	53
	Mep2 (P41948)	Sensor	*S. cerevisiae*	46

[a] *F. graminearum*, *Fusarium graminearum*.

synthetic medium containing 20 mM glutamine, due to the inability of the *areA* mutant to use ammonium as a nitrogen source. Following a 5-h starvation in a nitrogen-free medium, the mycelia were transferred to various media containing no nitrogen at all or 10 mM ammonium nitrate or glutamine as nitrogen sources. To find out whether the expression of the *mep* genes is controlled by the TOR kinase via AreA as has been already shown for the GA and bikaverin biosynthesis genes (47), we added 200 ng/ml rapamycin to one set of flasks.

All three *mep* genes are strongly repressed by ammonium and glutamine and derepressed under nitrogen starvation conditions (Fig. 3). The three *mep* genes were not expressed in the *areA* deletion strain under all of the growth conditions tested, and several single and double GATA or TATC sequence elements were found in the 5′ noncoding regions of all three genes (data not shown), which is consistent with the idea that the *mep* genes are directly regulated by AreA. Interestingly, the *mepA* and *mepB* genes are also not expressed in the Δ*glnA* mutant whereas *mepC* is upregulated in this mutant except for the medium with glutamine (Fig. 3).

Functional complementation assays in yeast. To determine whether the *F. fujikuroi mep* genes encode functional ammonium transporters, we tested the ability of the *F. fujikuroi* permeases to rescue the growth defect of an *S. cerevisiae* mutant (MLY131α) in which all three Mep-encoding genes had been deleted. All three *F. fujikuroi mep* genes were able to complement the yeast *mep1 mep2 mep3* triple mutant to restore growth on low-ammonium medium, consistent with these encoding functional ammonium permeases (Fig. 4). The level of growth was equal to the same strain transformed with the *S. cerevisiae MEP2* gene that was expressed from the same vector as the *F. fujikuroi mep* genes (Fig. 4).

The most striking phenotype of a fungal ammonium permease mutant is the loss of filamentous growth by *S. cerevisiae* and *C. albicans* mutant strains that lack the permease Mep2 (3, 25). Certain members of the AmtB/Mep2/Ump2/Rh family are able to complement the pseudohyphal defect of the *S. cerevisiae* Mep2 diploid mutant, e.g., the *U. maydis UMP2* and the *H. cylindrosporum AMT1* genes (18, 44). Therefore, we analyzed the extent to which the *F. fujikuroi mep* genes were able to restore the pseudohyphal growth defect of the *S. cerevisiae* diploid *mep2*Δ/*mep2*Δ strain. Transformants containing the individual *F. fujikuroi* genes were grown on low-ammonium medium for 6 days. The *mepA* and *mepC* permease genes were able to complement the *mep2*Δ/*mep2*Δ mutant under these conditions, with MepA being the stronger inducer of pseudohyphal growth. The *F. fujikuroi mepB* gene did not complement the *S. cerevisiae* mutant, notwithstanding the fact that it was expressed from the same promoter as the other two permease genes.

[^{14}C]methylamine uptake. To confirm that the *F. fujikuroi mep* genes encode functional ammonium permeases, we assayed their ability to mediate [^{14}C]methylamine uptake when expressed in a *S. cerevisiae* Mep-deficient mutant from a galactose-induced promoter. Cells were grown to mid-log phase using proline as a nitrogen source, and the rate of methylamine uptake over a range of methylamine concentrations was determined. All three *F. fujikuroi* Mep proteins mediated detectable methylamine uptake that increased with increasing concentrations of external methylamine. In the case of MepA and MepC, the permeases became saturated over the range of methylamine concentrations used (Fig. 5). MepA has the highest relative affinity for methylamine with an apparent K_m of 140 μM. MepC exhibited a lower affinity for methylamine with an apparent K_m of 1.37 mM and a higher V_{max} than MepA. Under these experimental conditions, the highest external concentrations of methylamine did not saturate MepB and precluded the determination of the kinetic parameters of this particular permease. We note that it was also not possible to determine the K_m of the low-affinity permease Ump1 from *U. maydis* using

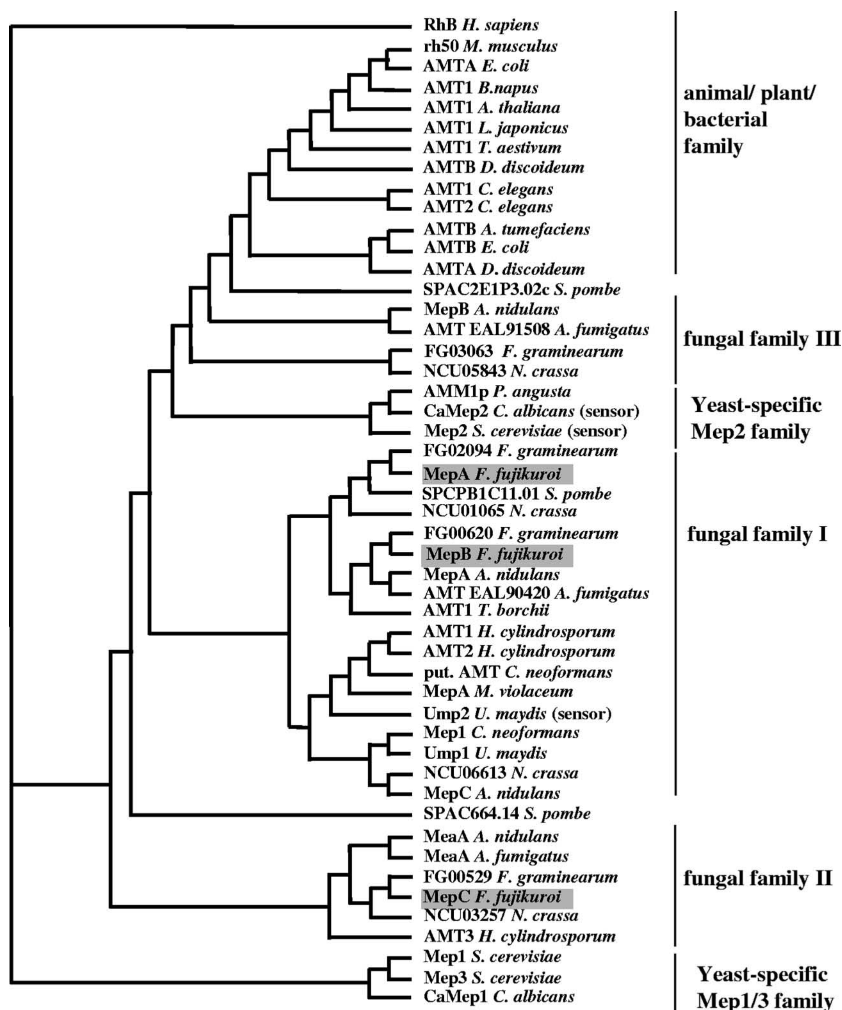

FIG. 1. Dendrogram of aligned protein sequences of known mammalian, plant, bacterial, and fungal permeases of the MEP/AMT family or putative ammonium permeases chosen by homology. The following are accession numbers for the indicated proteins: *Homo sapiens* RH type B, AAG01086; *Mus musculus* rh50, AAC25155; *Escherichia coli* AMTA, AAA97110; *Brassica napus* AMT1, AAG28780; *Arabidopsis thaliana* AMT1, P54144; *Lotus japonicus* AMT1, AAG24944; *Triticum aestivum* AMT1, AAS19466; *Dictyostelium discoideum* AMTB, BAB39710; *Caenorhabditis elegans* AMT1, P54145; *C. elegans* AMT2, Q20605; *Agrobacterium tumefaciens* AMTB, AAL43739, *E. coli* AMTB, AAD14837; *D. discoideum* AMTA, BAB39709; *S. cerevisiae* Mep1, P40260; *S. cerevisiae* Mep3, P53390; *H. cylindrosporum* AMT3, AAK82417; *Aspergillus nidulans* MeaA, EAL73117; *Aspergillus fumigatus* MeaA, EAL87679; *Schizosaccharomyces pombe* hypothetical protein SPAC664.14, CAB65815; *Cryptococcus neoformans* Mep1, AAW40795; *U. maydis* Ump1, AAL08424; *H. cylindrosporum* AMT1, AAM21926; *H. cylindrosporum* AMT2, AAK82416; *C. neoformans* putative ammonium transporter, AAW45844; *U. maydis* Ump2, AAO42611; *Microbotryum violaceum* MepA, AAD40955; *A. fumigatus* ammonium transporter, EAL90420; *A. nidulans* MepA, AAL73118; *F. fujikuroi* MepB; *T. borchii* AMT1, AAL11032; *N. crassa* MepA, CAD21326; *F. fujikuroi* MepA; *S. cerevisiae* Mep2, P41948; *Candida glabrata* unnamed protein, XP_447968; *C. albicans* hypothetical protein CaO19.13117, XP_713400; *Pichia angusta* AMM1p, AAQ76838; *A. fumigatus* ammonium transporter EAL91508; *Phytophthora infestans* ammonium transporter, AAN31513.

the same experimental system (44). We can conclude, therefore, that MepB has the lowest affinity and highest capacity for methylamine of the three *F. fujikuroi* ammonium permeases.

Generation and analysis of *mepA*, *mepB*, and *mepC* single and double deletion strains. Deletion of the three ammonium permease genes *mepA*, *mepB*, and *mepC* was performed by transforming the wild-type strain with the replacement cassettes of the vectors pΔmepA (hygromycin resistance marker), pΔmepB, and pΔmepC (both with the nourseothricin resistance cassette), respectively (Fig. 6) (see Materials and Methods). Altogether, three Δ*mepA* mutants (Δ*mepA*-T4, -T9, and -T10), three Δ*mepB* mutants (Δ*mepB*-T1, -T2, and -T10) and four Δ*mepC* mutants (Δ*mepC*-T4, -T11, -T17, and -T22) were obtained. Deletion mutants transformed with the same replacement cassette showed similar phenotypes whereas transformants with ectopic integrations of the replacement cassette behave as the wild type.

The growth of one deletion strain from each group was examined on plates with various ammonium concentrations (0 to 100 mM) and with 10 mM glutamine. The Δ*mepA* strain exhibited wild-type levels of growth on all ammonium concentrations. This is consistent with our data from [^{14}C]methylamine uptake experiments showing that this permease seems to have a minor role in ammonium acquisition not only in *S.*

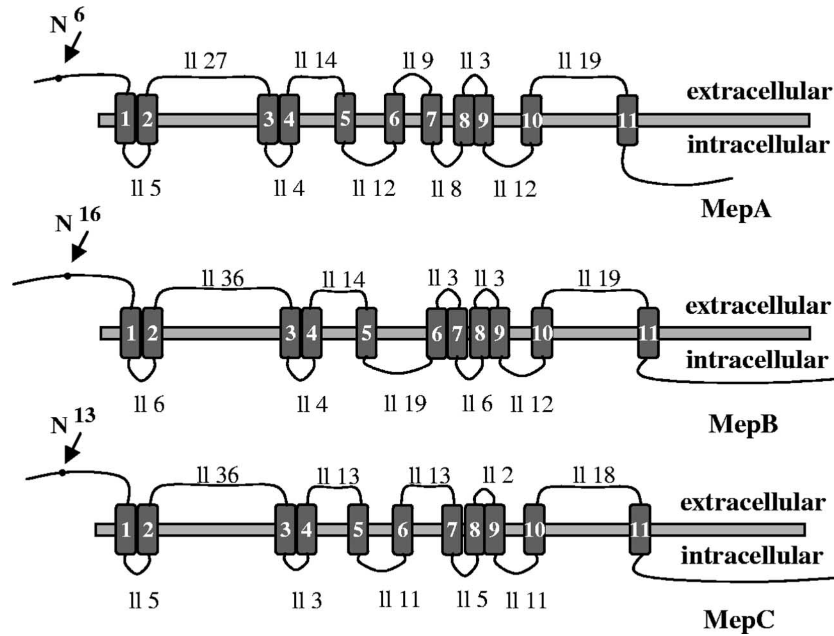

FIG. 2. Transmembrane structure of MepA, MepB, and MepC. Data were obtained from TMHMM2 (20). All three proteins consist of 11 transmembrane helices and 10 loops of denoted length (ll).

cerevisiae but also in *F. fujikuroi* and that the lack of MepA is fully compensated by MepB and MepC. The Δ*mepB* strain, and to a lesser extent the Δ*mepC* strain, exhibited a decreased growth rate on medium with decreasing levels of ammonium, which was most severe on plates that completely lacked ammonium. Under these latter conditions, the Δ*mepA* and Δ*mepC* strains were still able to form a thin mycelium, similar to that on nutrient-free water agar, in contrast to the Δ*mepB* strains. (Fig. 7A). This is consistent with the idea that MepB is the major ammonium transporter in *F. fujikuroi* under these growth conditions, with the elimination of this permease causing a strong starvation-induced growth inhibition, probably due to a significant defect in ammonium transport. Surprisingly, 100 mM ammonium revealed a rather inhibitory effect on

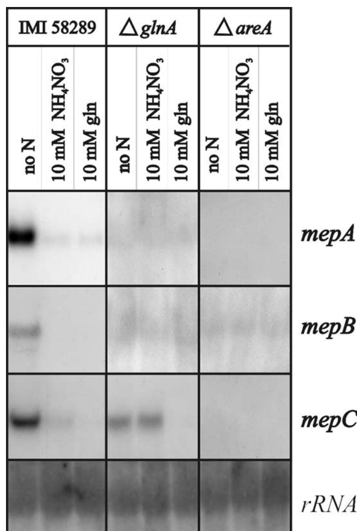

FIG. 3. Expression analysis of the *F. fujikuroi* ammonium permease genes *mepA*, *mepB*, and *mepC* in the wild-type IMI58289 and the Δ*glnA* and Δ*areA* mutant strains. The fungal strains were cultivated for 5 days in ICI medium with 10 mM glutamine, and after a 2-h incubation in nitrogen-free ICI medium, the washed mycelia were shifted into medium without nitrogen, with 10 mM ammonium nitrate, or with 10 mM glutamine.

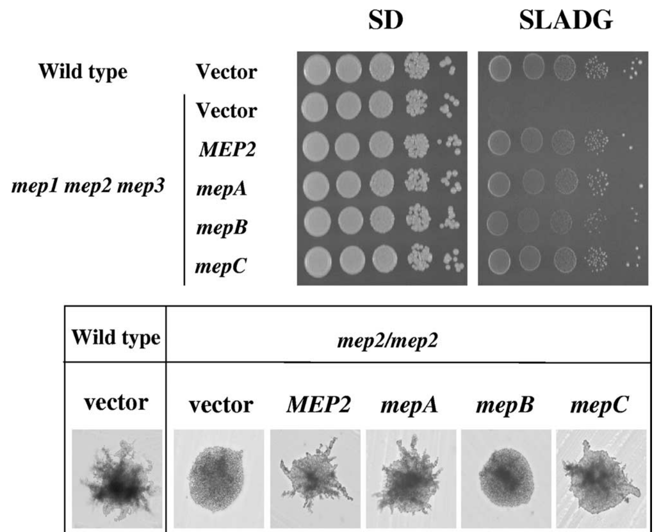

FIG. 4. Complementation of the yeast *mep1 mep2 mep3* triple mutant with the *F. fujikuroi mepA*, *mepB*, and *mepC* cDNA fragments under the control of the *S. cerevisiae GAL1* promoter. All three *mep* genes fully restored the growth of the *mep1 mep2 mep3*/*mep1 mep2 mep3* mutant on SLADG minimal medium. Transformation of the diploid yeast *mep2*/*mep2* mutant with the *F. fujikuroi mepA* and *mepC* genes, but not with the *F. fujikuroi mepB* gene, restored pseudohyphal growth on the nitrogen limited medium SLADG.

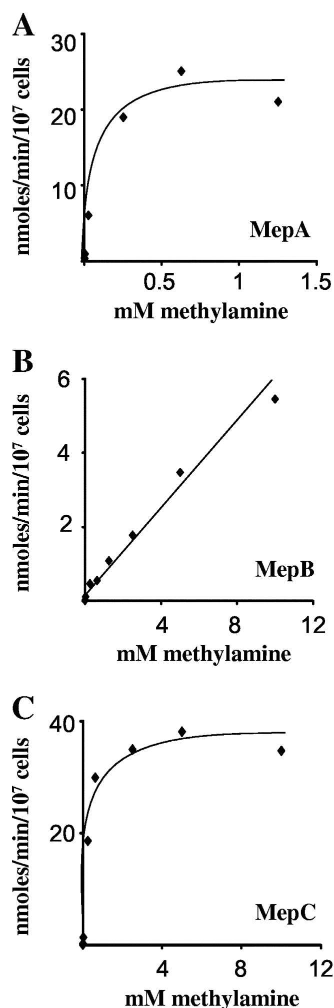

FIG. 5. The Mep proteins of *F. fujikuroi* mediate [^{14}C]methylamine uptake. Rate of uptake versus substrate concentration curves for MepA (A), MepB (B), and MepC (C) when proteins are expressed from the *GAL1-10* promoter in a *S. cerevisiae mep1 mep2 mep3* triple mutant strain (see Material and Methods).

growth of the wild type and the mutants; the reasons for this growth inhibition are not yet clear.

To test the combinational effects of the loss of multiple ammonium permeases, we generated Δ*mepA* Δ*mepB* and Δ*mepB* Δ*mepC* double mutants. Both the Δ*mepA* Δ*mepB* and the Δ*mepB* Δ*mepC* strains exhibited no further reduction in growth rate under low-ammonium conditions compared with the Δ*mepB* single mutant. The lack of increased sensitivity to low-ammonium growth by the additional deletion of either Δ*mepA* or Δ*mepC* confirmed the major role of MepB. The minimal growth of the Δ*mepB* Δ*mepC* strain under these conditions is probably attributed to nonspecific ammonium uptake and the presence of MepA. We were not able to construct a triple *mepA mepB mepC* mutant due to the lack of a suitable third resistance marker for *F. fujikuroi*. Therefore, the extent of nonspecific ammonium uptake cannot be precisely determined.

To quantify the growth defect of the mutants lacking one or two Mep permeases, we grew the wild type and the single and double mutants in liquid synthetic medium with ammonium sulfate (10 mM) or glutamine (10 mM) for 48 h and determined the dry weight of the mycelia. While all strains produce almost the same biomass when grown with glutamine as the nitrogen source, the Δ*mepB* strain and Δ*mepA* Δ*mepB* and Δ*mepB* Δ*mepC* double mutants produced significantly fewer mycelia when grown on ammonium (Fig. 7B). To link the phenotype of the Δ*mepB* mutants with the deletion of the *mepB* permease gene, we complemented the mutant Δ*mepB*-T10 with the *mepB* wild-type copy cloned into the vector pGPC1. Hygromycin-resistant transformants were screened for their ability to grow on low-ammonium-containing medium (1 mM ammonium citrate) and for the integration of the complementing *mepB* gene. Five of the 12 transformants tested revealed the expected PCR fragment for the *mepB* gene, and these transformants exhibited almost the same growth as the wild type and showed wild-type-like expression of the *mepB* gene (data not shown). Thus, the growth defect of Δ*mepB* mutants on low ammonium is clearly linked to the deletion of the *mepB* ammonium permease gene.

Deletion of *mepB* but not *mepA* or *mepC* confers highly deregulated secondary metabolism. To date, the impact of single ammonium transporters on secondary metabolism has not been studied in filamentous fungi. In *F. fujikuroi*, two different nitrogen-free metabolites, the GAs and the red pigment bikaverin, are strictly regulated by nitrogen metabolite repression. To test if the *F. fujikuroi* ammonium permeases influence GA and bikaverin biosynthesis, we analyzed the production of these secondary metabolites in the *mep* deletion strains. In wild-type cells, the initiation of bikaverin biosynthesis is a marker for nitrogen depletion, and the production of the pink pigment is accompanied by induction of GA biosynthesis (23, 50).

Wild-type and mutant strains were grown for 48 h in a medium containing 10 mM glutamine to support equal growth for all strains. Aliquots were inoculated into synthetic ICI medium containing 20 mM ammonium or glutamine. The color of the cultures was monitored at 15, 24, 48, and 72 h. The color of the wild-type and Δ*mepA* cultures changed from white (up to 24 h) to light pink (30 h to 48 h) to yellowish pink (72 h) (Fig. 8). In contrast, the single Δ*mepB* and the double Δ*mepA* Δ*mepB* and Δ*mepB* Δ*mepC* mutant strains displayed a red pigmentation already 15 h after inoculation that increased with longer incubation (Fig. 8). The pigmentation of the *mepC* deletion strain was also apparent after 15 h of cultivation but was not as intense as in strains that lacked *mepB*. Surprisingly, a slightly earlier pigmentation of Δ*mepB*, Δ*mepA* Δ*mepB*, and Δ*mepC* mutants was also observed in cultures with glutamine as nitrogen source (Fig. 8).

To identify the extent to which GA production in *F. fujikuroi* is also regulated by the ammonium permeases, we monitored the accumulation of GA3 in the wild-type and mutant strains. In a synthetic medium with 20 mM ammonium, the wild-type produced no GAs up to the full depletion of nitrogen sources in the medium but accumulated high amounts of these products under starvation conditions (33). The Δ*mepA* and *mepC* mutants behaved as the wild type: these strains did not produce visible amounts of GAs at 30 and 48 h. However, the Δ*mepB* strain and the Δ*mepA* Δ*mepB* and Δ*mepB* Δ*mepC* double mutants had already initiated GA3 production by 30 h (Fig. 8).

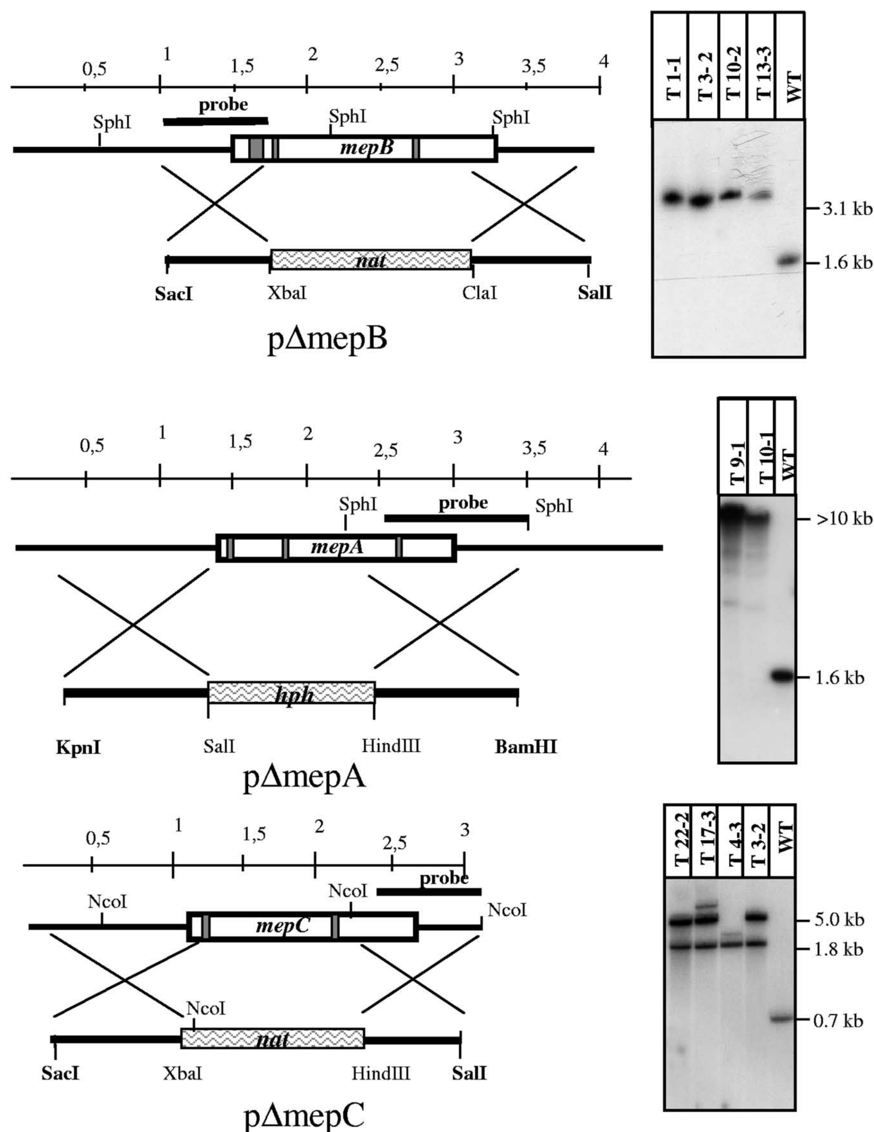

FIG. 6. Strategy for generating the replacement vectors pΔmepA, pΔmepB, and pΔmepC which were used for deletion of the corresponding ammonium permease genes. *mepB* and *mepA* were replaced by the nourseothricin and *mepA* by the hygromycin resistance cassettes. Introns are shown as gray bars. One of the flanks of each replacement vector was used as a probe for hybridization in Southern blotting. For confirmation of homologous integration of the replacement cassette into the corresponding *mep* locus, the genomic DNA of the wild type and the deletion mutants was digested with SphI (*mepA* and *mepB*) or with EcoRI (*mepC*). In all three cases, the loss of the wild-type (WT) fragment is shown.

With glutamine, all strains started to produce GA_3 only at 72 h (Fig. 8). Thus, deletion of *mepB* affects the biosynthesis of both secondary metabolites in a similar fashion. The early bikaverin and GA formation in the medium containing ammonium confirms the importance of MepB in ammonium uptake and cellular nitrogen homeostasis.

Gene expression of secondary metabolism genes is deregulated in *mepB* deletion strains. We analyzed the expression of GA and bikaverin biosynthesis genes using the same cultivation conditions that had been used for monitoring bikaverin and GA production. In medium containing 20 mM ammonium, the bikaverin biosynthetic genes *pks4*, *MT*, and *MO* (encoding the polyketide synthase, a methyltransferase, and a monooxygenase from the bikaverin gene cluster) (23; also P. Wiemann and B. Tudzynski, unpublished data) and the GA biosynthetic genes (*cps/ks* and *P450-1*) (reviewed in reference 51) were expressed by 24 h in the *mepB* single and double mutants. The same transcripts were detectable only at 48 or 72 h in the wild-type and the *mepA* and *mepC* mutant strains (Fig. 9). This pattern of expression has also been observed for other *areA* target genes, e.g., the peptide transporter gene *mtd1* and the amino acid permease gene *aap8* (B. Schönig and B. Tudzynski, unpublished data; also data not shown) as well as a set of GS target genes, e.g., *ddr48* and *cipC* (Fig. 9), which are not expressed in the *glnA* mutant (46). As expected, nitrogen deregulation is most significant when the cultures contain am-

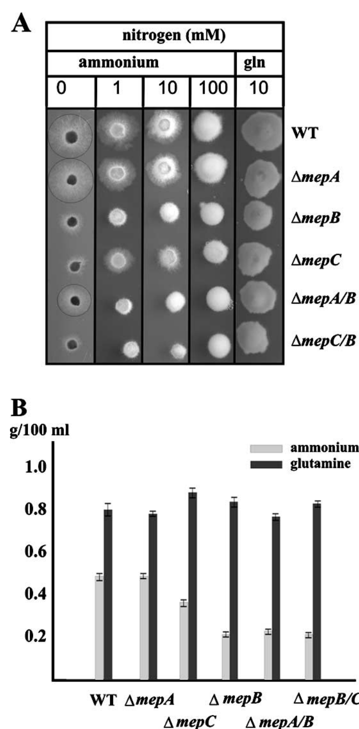

FIG. 7. Plate assays for monitoring the growth of the wild-type and mutant strains on ICI medium with different ammonium citrate concentrations. (A) Growth of single and double ammonium permease deletion strains on different ammonium citrate concentrations (0, 1, 10, and 100 mM). The circles around the wild type (WT), *mepA*, and *mepC* mutants show the borders of the colonies on plates without any nitrogen. *mepB* single and Δ*mepA* Δ*mepB* (Δ*mepA*/*B*) and Δ*mepB* Δ*mepC* (Δ*mepB*/*C*) double mutants are not able to grow on this medium. (B) Biomass production of the wild type (WT) and the five mutant strains in ICI medium with 10 mM ammonium sulfate or 10 mM glutamine after 72 h of incubation.

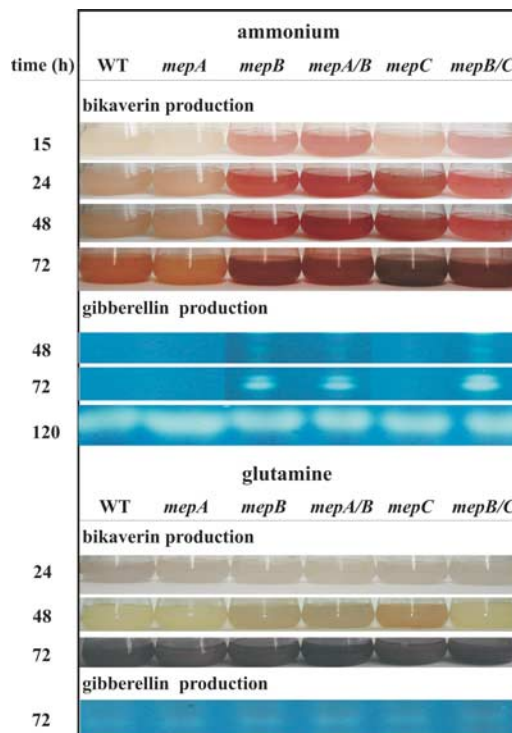

FIG. 8. Production of bikaverin and GA3 by the wild-type strain IMI58289; the Δ*mepA*, Δ*mepB*, and Δ*mepC* single mutants; and the Δ*mepA* Δ*mepB* (*mepA*/*B*) and Δ*mepB* Δ*mepC* (*mepB*/*C*) double mutants. The strains were cultivated in a time course for 3 days in medium containing 12 mM ammonium sulfate or 12 mM glutamine. The pigmentation of the culture fluids demonstrates the much earlier start of the bikaverin production in the Δ*mepB* single and double mutants and also in the Δ*mepC* mutant with ammonium as the nitrogen source. The GA3 production under the same conditions is shown by TLC. As for the bikaverin production, the GAs were produced much earlier (at 30 h) in the Δ*mepB* single and double mutants, whereas the production starts only at 90 h in the wild type and Δ*mepA* mutant.

monium as the nitrogen source. However, the expression of the GA and bikaverin biosynthetic genes is also partially deregulated in single and double *mepB* mutants and, for some genes, also in *mepC* mutants with glutamine as a nitrogen source, although the effect is not as clear as with ammonium (Fig. 9).

Is ammonium incorporation by metabolism required for nitrogen metabolite repression? Fungal ammonium assimilation occurs via its incorporation into glutamate and glutamine by glutamate dehydrogenase A (GdhA) and GS, respectively (38). To answer the question whether ammonium itself acts as an effector of nitrogen metabolite repression, or if repression of GA and bikaverin biosynthesis genes (and other NCR genes) by ammonium is due to its conversion to glutamate and/or glutamine, we completely abolished ammonium incorporation into metabolism. For this, we used a *gdhA* deletion mutant (B. Tudzynski, unpublished data) which is unable to assimilate ammonium through reductive synthesis of glutamate from 2-oxoglutarate, and we inhibited GS with the specific inhibitor MSX to abolish the second route of ammonium assimilation by GS. We compared the expression of the three *mep* genes and the GA and bikaverin biosynthesis genes in the *gdhA* mutant with that in the wild type in medium without nitrogen and with ammonium, with or without MSX. Under these conditions, MSX did not prevent strong repression of gene expression by ammonium either in the wild type or in the *gdhA* mutant (Fig. 10). However, under starvation conditions (no nitrogen), several genes were upregulated in the *gdhA* mutant, suggesting that they are inhibited by both ammonium and glutamate/glutamine in the wild type. Interestingly, *mepC* is slightly induced by intracellular ammonium in the mutant, and this induction is abolished by MSX (Fig. 10). On the other hand, the expression of *mepB* is slightly induced in the mutant when MSX is added to the ammonium-containing medium, suggesting a functional link between MepB and the GS. In summary, these results demonstrate that ammonium itself causes strong nitrogen metabolite repression by transducing the signal of nitrogen availability.

Does MepB play a regulatory role? The strong effect of *mepB* deletion on expression of secondary metabolite and some other NCR genes (e.g., *mtd1* and *aap8*) can be explained by its role as the major ammonium permease or by a suggested additional role as a sensor-like regulatory component transducing the signal of nitrogen availability via a yet unknown signaling pathway to AreA. To answer this question, we overexpressed *mepC* (which was shown to be a high-capacity per-

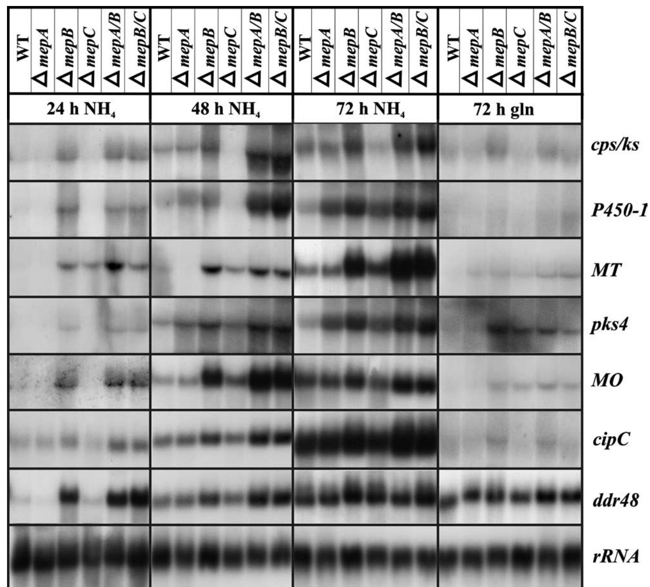

FIG. 9. Northern blot analysis with the *F. fujikuroi* wild type (WT); the Δ*mepA*, Δ*mepB*, and Δ*mepC* single mutants; and the Δ*mepA* Δ*mepB* (Δ*mepA*/*B*) and Δ*mepB* Δ*mepC* (Δ*mepB*/*C*) double mutants. Strains were cultivated in a time course for 3 days in medium containing 12 mM ammonium sulfate or glutamine. For the glutamine cultures, only one time point (72 h) is shown due to the low expression levels of the GA and bikaverin genes at 24 and 48 h. The filters were probed with the cDNA fragments of the genes.

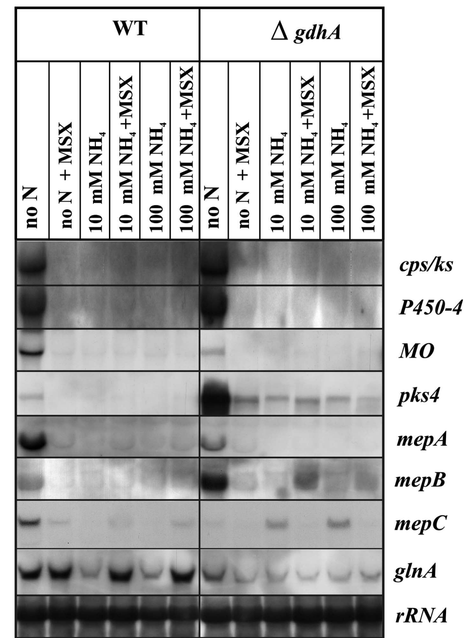

FIG. 10. Northern blot analysis with the *F. fujikuroi* wild type (WT) and Δ*gdhA* mutant. Strains were cultivated in a time course for 3 days in medium containing 10 mM ammonium sulfate. The mycelia were washed and shifted for 2 h into nitrogen-free medium or medium containing 10 or 100 mM ammonium sulfate, with or without the GS inhibitor MSX (2 mM). The filters were probed with the cDNA fragments of the GA (*cps*/*ks* and *P450-4*) and bikaverin (*MO* and *pks4*) biosynthetic genes as well as the three *mep* genes and the GS-encoding gene *glnA*.

mease beside MepB) in the *mepB* mutant to rescue the poor growth of *mepB* mutants on ammonium. We fused the *mepC* gene with the strong promoter of the *F. fujikuroi glnA* gene ($glnA_{prom}$) (46) and cloned the chimeric fragment into vector pUCH2-8 containing the hygromycin resistance cassette. The resulting vector pglnA::mepC was used to transform the *mepB* mutant T1. Hygromycin-resistant transformants were evaluated by PCR for the presence of the $glnA_{prom}$-*mepC* fusion product and by growth assays on medium with 1 mM ammonium. Six out of 12 transformants contained the PCR product and exhibited enhanced growth compared to the *mepB* mutant, whereas the other transformants (e.g., T5) did not contain the fusion product and grew as poorly on 1 mM ammonium as the Δ*mepB* recipient strain (Fig. 11A). Statistical analysis of the growth rate of the *mepC*-overexpressing strains compared with that of the *mepB* mutant and the wild type is shown in Table 2.

If the strong phenotype of *mepB* mutants regarding the derepression of secondary metabolism genes is due to ammonium limitation, we would expect a loss of this deregulation by overexpressing *mepC*. We cultivated the wild type, the *mepB* mutant, and four *mepC*-overexpressing transformants (designated Δ*mepB*+*mepC*-1, -2, -3, and -10) in ICI medium with 20 mM ammonium sulfate. Surprisingly, the *mepC*-overexpressing mutants already showed a strong pigmentation after 15 h, similar to the *mepB* mutant despite having a wild-type growth pattern (Fig. 11B). The mycelia were harvested after 24 h and used for expression studies by Northern blot analysis. The *mepC* gene was strongly up-regulated in the mutants, as expected. The GA and bikaverin genes are highly expressed at 24 h as is the *mepB* mutant, despite the almost full restoration of growth (Fig. 11C). These data suggest that MepB plays an additional role in nitrogen regulation in *F. fujikuroi*, probably by sensing and transducing the signal of ammonium availability.

DISCUSSION

Transport capacity of MepA, MepB, and MepC. In this study, we report the cloning and characterization of three genes, *mepA*, *mepB*, and *mepC*, which encode ammonium permeases in *F. fujikuroi*. All three proteins share a high level of sequence identity to other ammonium permeases of the AmtB/Mep family and are able to complement a *S. cerevisiae mep1 mep2 mep3* triple mutant despite the evolutionary distance between *S. cerevisiae* and *F. fujikuroi*. Our data indicate that they differ in ammonium transport capacity and affinity toward ammonium ions. MepB appears to be the major ammonium transporter in *F. fujikuroi*, followed by MepC, as their deletion resulted in substantially reduced growth on medium containing 1 mM or 10 mM ammonium. These data correspond to the [^{14}C]methylamine uptake data in *S. cerevisiae* by which MepB was shown to have the lowest affinity and highest capacity for methylamine of the three *F. fujikuroi* ammonium permeases. A similar phenotype has been described for *meaA* deletion mutants of *A. nidulans*, which exhibit a significantly reduced growth rate on medium with low concentrations of ammonium, are resistant to methylamine, and play a physiological role in retaining the ammonium derived from the metabolism of other

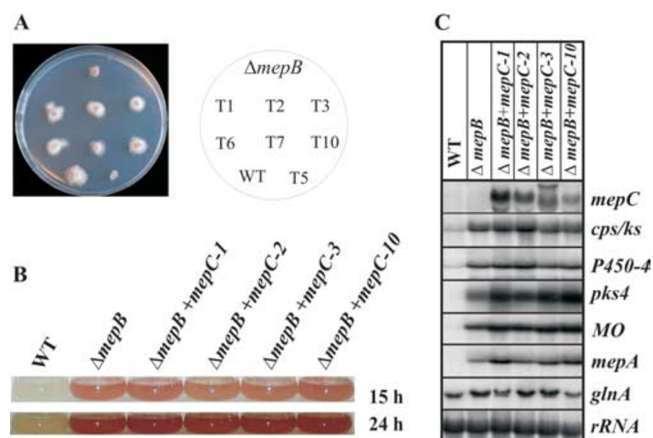

FIG. 11. Overexpression of mepC ($glnA_{prom}$::*mepC*) in the Δ*mepB* deletion mutant. (A) Growth of several transformants carrying the overexpression cassette on 1 mM ammonium sulfate. Transformants T1, T2, T3, and T10 were chosen for detailed analyses (see below). Transformant T5 does not contain the whole promoter-gene fusion fragment and shows the same growth defect as the Δ*mepB* deletion mutant (negative control). (B) Bikaverin production in the wild type, Δ*mepB* mutant, and four *mepC*-overexpressing transformants, which still show a much earlier pigment production than the wild type. (C) Northern blot analysis with the *F. fujikuroi* wild type, the Δ*mepB* mutant, and four *mepC*-overexpressing transformants. The strains were cultivated for 24 h in medium containing 12 mM ammonium sulfate. The early pigmentation (as shown in panel B) corresponds very well with early expression of several AreA target genes (for an explanation, see the legend of Fig. 10) in the *mepC*-overexpressing transformants. WT, wild type.

nitrogen sources (34). Increased methylamine resistance has not been observed for *F. fujikuroi mep* mutants due to the extremely high resistance of the wild type. Our analysis of the relative affinities of the *F. fujikuroi* Mep permeases clearly shows that these proteins are able to mediate methylamine uptake. This suggests that *F. fujikuroi* contains an efficient mechanism to detoxify imported methylamine. Consistent with this, the *F. fujikuroi* genome contains at least three genes that code for homologues of fungal amine oxidases which are known to catalyze the oxidative deamination of amines and can therefore confer resistance to methylamine (11). The differential number, expression levels, and/or specificity of amine oxidases could result in a range of sensitivity to methylamine among different fungi species.

Interestingly, the MepB permease of *F. fujikuroi* does not group together with MeaA or yeast Mep1, despite their functional similarity as high-capacity permeases. Instead, MepB groups with the *A. nidulans* and *Tuber borchii* high-affinity permeases MepA and TbAMT1, respectively (34, 37). MeaA shows high sequence similarity to the *F. fujikuroi* MepC permease, which is also a high-capacity permease according to the reduced growth on medium containing 1 to 100 mM ammonium. In contrast to *mepB* and *mepC* mutants, *mepA* mutants exhibit no obvious phenotype. They grow at the same rate as the wild type and produce colonies with thin mycelia, even on plates without nitrogen. Analysis of methylamine uptake confirmed that MepA is a high-affinity and low-capacity permease. MepA groups into an AmtB/Mep subclass together with the *A. nidulans* MepA and the *F. fujikuroi* high-capacity permease MepB. Therefore, the two *F. fujikuroi* permeases MepA and MepB are phylogenetically highly related but differ in their affinity for ammonium and their transport capacity, demonstrating that a high degree of sequence similarity cannot be strictly correlated with functional inferences.

The expression of *mepA*, *mepB*, and *mepC* is highly dependent on AreA. The expression of all three *mep* genes is uniformly regulated by AreA. In the Δ*areA* mutant, no transcript levels were detected for *mepA*, *mepB*, and *mepC*, consistent with the finding that the *F. fujikuroi* Δ*areA* mutant does not grow on medium with ammonium as the only nitrogen source. This contrasts with *A. nidulans* where disruption of the zinc finger region of *areA* does not result in loss of ability to grow on ammonium but prevents utilization of nitrogen sources other than ammonium and glutamine (21).

The expression of several Mep2-type permeases, such as the *S. cerevisiae MEP* genes (30) and the four permeases in *A. nidulans* (34, 35, 36), is nitrogen regulated and depends on activation by the transcription factors Gln3 and AreA, respectively. Genes encoding permeases of the Mep1/Mep3 type, such as Mep1 in *C. albicans* (3) and MeaA in *A. nidulans* (34, 35), are expressed at much lower levels than the high-affinity Mep2-type transporters. In *F. fujikuroi*, *mepA* shows the highest expression level similar to other Mep2-like permeases. Interestingly, the expression of *mepC* is upregulated in the *glnA* mutant in medium without nitrogen or with 10 mM ammonium, whereas glutamine overcomes this effect. In contrast, the *mepA* and *mepB* genes are repressed in both the *areA* and *glnA* mutants. Further studies will be required to identify the mechanism of GS-dependent expression of the *mep* genes.

Does MepB participate in nitrogen regulation? Some fungal ammonium permeases have evolved a regulatory function. In *S. cerevisiae*, several plasma membrane-localized permeases are able to sense the environmental availability of amino acids, ammonium, and sugars (10, 52). Among the three Mep proteins, Mep2 is a receptor for ammonium and is required for pseudohyphal growth under nitrogen starvation conditions (25). In *C. albicans*, CaMep2, but not CaMep1, also induces filamentous growth under limiting nitrogen conditions via the activation of both mitogen-activated protein kinase and the Ras1-cyclic AMP-dependent pathways (3). The maize pathogen *U. maydis* expresses two ammonium permease-encoding genes, *ump1* and *ump2* (44). Ump2 is required to trigger filamentous growth on low-nitrogen medium and complements the pseudohyphal growth defect of the *S. cerevisae mep2* mutant, consistent with a sensor role (44). Similarly, it has been

TABLE 2. Partial restoration of vegetative growth of the Δ*mepB* mutant by overexpression of MepC

Strain	Colony diam (mm) at[a]:			
	48 h	72 h	96 h	168 h
Wild type	11 ± 1	14 ± 0	16 ± 1	22 ± 2
Δ*mepB*-T1	6 ± 1	8 ± 1	9 ± 1	11 ± 1
Δ*mepB*+*mepC*-1	9 ± 1	12 ± 1	14 ± 2	21 ± 2
Δ*mepB*+*mepC*-2	9 ± 1	12 ± 1	13 ± 1	21 ± 2
Δ*mepB*+*mepC*-3	9 ± 1	12 ± 1	14 ± 1	22 ± 2
Δ*mepB*+*mepC*-10	9 ± 1	13 ± 1	13 ± 2	21 ± 2

[a] Data are the average of three independent measurements ± standard deviation.

proposed that in the mycorrhiza fungus *H. cylindrosporum*, the high-affinity ammonium transporters Amt1 and Amt2 induce, via an as yet unknown signal transduction cascade, a switch in the fungal growth mode during the formation of mycorrhiza (17).

The molecular mechanisms involved in transporter-mediated ammonium regulation are not presently known although some important aspects have been identified in yeast that may be conserved within fungi. The Mep2-like permeases are high-affinity transporters and are the most highly transcribed in response to ammonium limitation. Promoter exchange and reporter gene experiments show that CaMep2 needs to be expressed at very high levels to mediate filamentous growth induction under low-ammonium conditions (3). A mutant strain lacking such a regulatory permease would also be expected to exhibit a signaling phenotype, and no phenotype that could be attributed to a reduction in nitrogen availability, such as a reduction in growth rate. Other characteristics of the yeast ammonium permeases are less predictive with regard to a role as regulatory proteins. Sequence alignments of the currently available Meps in the database show that fungal Meps contain a putative protein kinase A (PKA) phosphorylation site [RRX(S/T), where X is any residue] that is not present in those from other organisms (44). Point mutations in these sites in Ump2 (Ser288-Ala) and Mep2 (Thr288-Ala) do not abolish ammonium transport, but both mutant Meps fail to complement the pseudohyphal growth defect of the *S. cerevisiae mep2* mutant (44). However, nonsensing homologues of Mep2 (e.g., Mep1 and Mep3 of *S. cerevisiae*) also contain a similar potential PKA phosphorylation site. In addition, the *S. cerevisiae* Mep2 is glycosylated at the fourth amino acid. However, Mep2^{N4Q} mutation of the *S. cerevisiae* glycosylation site does not prevent pseudohyphal growth (32). Many of the Mep2-specific features, such as glycosylation and putative PKA phosphorylation sites, are found in all three *F. fujikuroi* Mep proteins. In addition, all *Fusarium* Meps are predicted to have the same overall structure as Mep2, including the putative sensing loop (25). It is therefore challenging to identify conserved characteristics within the ammonium permeases of filamentous fungi that define a regulatory function.

The data presented in this study are most consistent with MepA having the characteristics of a regulatory permease: the gene is highly expressed, the *mepA* mutants show no severe growth defect on low ammonium, MepA (but also MepB and MepC) is predicted to be glycosylated, and MepA is a high-affinity transporter that is able to induce pseudohyphal growth in a *mep2*-deficient *S. cerevisiae* mutant. MepC also restores the pseudohyphal growth in the yeast *mep2* mutant, but the deletion of *mepC* conferred an obvious growth phenotype in *F. fujikuroi*. However, the expression patterns of the AreA target genes argue against a role of MepA or MepC as extracellular ammonium sensors that link ammonium availability to the regulation of AreA activity. If MepA or MepC were a sensor permease, then the signal for nitrogen sufficiency would be transduced in Δ*mepB* mutants, resulting in strong repression of AreA target genes, but this is not the case.

In contrast, MepB does not restore pseudohyphal growth of the *S. cerevisiae mep2* mutant, and *mepB* mutants do not grow under low-ammonium conditions, making it appear to be a less likely candidate for a regulatory permease. However, MepB is the only permease affecting the regulation of AreA target genes, such as the GA and bikaverin biosynthesis genes, which are derepressed in the Δ*mepB* mutant under ammonium-sufficient conditions. In addition, a partial derepression of GA and bikaverin genes in the *mepB* mutants was observed also on glutamine, similar to *C. albicans* where the Mep2 orthologue is required for nitrogen limitation-induced filamentation under conditions of both ammonium and amino acid limitation (3).

There are two possible explanations for the strong impact of MepB on regulation. First, the effects on gene expression may be due to reduced ammonium transport, in agreement with the growth defect of the *mepB* mutant and with methylamine uptake experiments demonstrating that MepB is the major ammonium transporter in *F. fujikuroi*. Second, MepB might play an additional role in nitrogen regulation despite not exhibiting a regulatory function in the heterologous *S. cerevisiae* model. If the derepressing effect on nitrogen-regulated genes in the Δ*mepB* mutant is the result of ammonium limitation due to the loss of the major transporter, overexpression of the second high-capacity permease, MepC, should restore not only the wild-type growth but also the wild-type expression levels of these genes. However, despite the significantly increased growth of the *mepC*-overexpressing mutants (Fig. 11), the deregulation of GA and bikaverin biosynthesis genes as well as other AreA-dependent genes is the same as in the Δ*mepB* mutant. These data support our suggestion that MepB might play an important role in transport and sensing of ammonium although this permease does not complement the pseudohyphal growth defect in yeast. However, due to the evolutionary distance between *F. fujikuroi* and *S. cerevisiae*, heterologous expression of *F. fujikuroi mep* genes need not necessarily mimic all of the regulatory events or expression levels that occur in the natural host.

However, more definitive evidence will be required before a sensing function can be attributed to any of the *F. fujikuroi* ammonium permeases. Such evidence includes the isolation of mutants, such as those recently identified in Mep2 of *S. cerevisiae* (J. Rutherford et al., personal communication), that separate the transport and sensing function of the permease, a demonstration of interactions with signal transduction proteins, or the identification of downstream physiological events such as altered transcriptional programs. This will also include the identification of mutants analogous to the hyperactive $\text{Mep2}^{\text{G349C}}$ mutant of *S. cerevisiae* that restores dimorphic growth in a pseudohyphal-deficient mutant (*npr1*Δ) and thereby establishes a clear correlation between Mep2 dependent transport and signaling (4).

Is the metabolism of ammonium essential for triggering nitrogen repression? In *S. cerevisiae*, the stimulus for the expression of nitrogen-regulated genes by the AreA homologue Gln3 is a drop in the intracellular level of glutamine (7, 27). Therefore, we sought to establish whether the reduced intracellular glutamine level, resulting from the compromised ammonium transport capacity in the *mepB* mutants, or the decreased ammonium availability itself triggers the activation of AreA via a yet unknown signaling cascade and the derepression of AreA target genes. To answer this question, we inactivated both the GdhA and GS pathways for ammonium in-

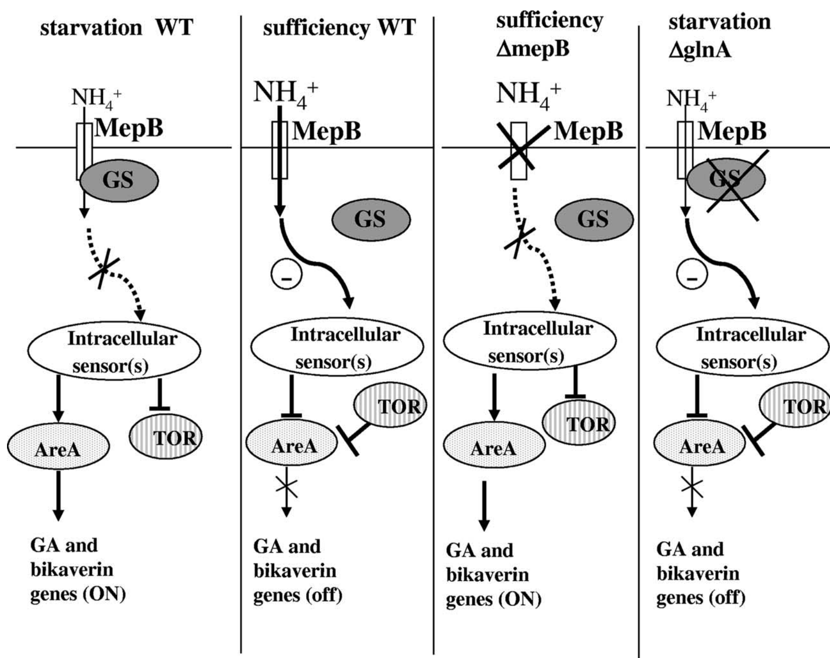

FIG. 12. Model of the nitrogen regulation network in *F. fujikuroi*. MepB is postulated as a key component in this model, acting as the major ammonium permease. In addition, *mepB* might also act as a nitrogen sensor either by mediating the signal of nitrogen availability or indirectly by causing a drop in the intracellular glutamine level. In the latter case we suggest an intracellular sensor transducing the glutamine signal to AreA via TOR and/or additional signaling pathways. WT, wild type.

corporation into metabolism. For this, we used a *gdhA* deletion mutant and treated the mycelium with the specific GS inhibitor MSX. Ammonium-induced strong repression of GA and bikaverin biosynthetic genes was not prevented under these conditions, demonstrating that the intracellular level of glutamine is not the only signal sensed by the fungal cells that activates/inactivates signaling pathways to AreA and that ammonium itself is an effector of nitrogen metabolite repression in *F. fujikuroi*.

Conclusions. We identified one of the three ammonium permeases, MepB, as the major transporter and as a potential element in the *F. fujikuroi* nitrogen regulation network. Although the inability of MepB to restore the pseudohyphal growth defect of *S. cerevisiae mep2* mutants argues against a direct role of MepB as a regulator, at least in a heterologous organism, several other features support such a role. The strongest argument for a regulatory role is the overexpression of *mepC* encoding the second high-capacity transporter in *F. fujikuroi*, which resulted in significantly improved growth but did not prevent the deregulated expression pattern of the nitrogen-regulated secondary metabolism genes.

Recently, we showed that GA and bikaverin biosynthetic genes are strongly repressed in a Δ*glnA* mutant even under nitrogen-limiting conditions, despite the fact that the intracellular glutamine pool is drastically reduced. These results are in contrast to our expectation and suggest a regulatory role of GS in addition to its enzymatic function, e.g., by protein-protein interactions with other components of the nitrogen regulation network (46). Summarizing our current knowledge of the role of GS and other components involved in nitrogen regulation of secondary metabolism in *F. fujikuroi* and the postulated regulatory role of MepB, we propose a model for nitrogen sensing and signaling (Fig. 12). In this model, MepB is the major ammonium transporter which is able to sense ammonium (nitrogen) availability and to transduce a repressing signal to AreA by an as yet unknown signaling pathway. Under ammonium limitation conditions, GS binds to MepB, thereby blocking the transduction of this repressing signal and resulting in the induced expression of *glnA* and probably increased GS levels. The missing signal leads to an activation of AreA and derepression of AreA target genes, such as the *mep* and the GA and bikaverin biosynthetic genes. This model can also explain why GS is needed for the expression of the GA and bikaverin biosynthesis genes under nitrogen starvation conditions (46). The inability of GS to bind to MepB in the *glnA* deletion mutant would enable the putative sensor MepB to constitutively activate signaling, resulting in strong repression of AreA target genes. In support of this model, a close metabolic coupling of the membrane-bound GS to the ammonium channel AmtB has been recently shown for *E. coli* (19). This model will provide the basis of future work to identify the molecular mechanisms that link nitrogen availability to the regulation of secondary metabolism in *F. fujikuroi*.

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