

PcMtr, an aromatic and neutral aliphatic amino acid permease of *Penicillium chrysogenum*[☆]

Hein Trip, Melchior E. Evers, Arnold J.M. Driessen*

Department of Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

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Abstract

The gene encoding an aromatic and neutral aliphatic amino acid permease of *Penicillium chrysogenum* was cloned, functionally expressed and characterized in *Saccharomyces cerevisiae* M4276. The permease, designated PcMtr, is structurally and functionally homologous to Mtr of *Neurospora crassa*, and unrelated to the Amino Acid Permease (AAP) family which includes most amino acid permeases in fungi. Database searches of completed fungal genome sequences reveal that Mtr type permeases are not widely distributed among fungi, suggesting a specialized function.

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1. Introduction

Fungi possess a number of permeases for the uptake of amino acids, which can serve as nitrogen and/or carbon source or as building blocks for protein and peptide synthesis. These permeases have different properties with respect to substrate specificity, affinity, capacity and regulation [1,2]. Most fungal amino acid permeases belong to a unique transporter family, referred to as the Amino Acid Permease (AAP) family [3] or the yeast amino acid transporter (YAT) family [4], which is a subfamily of the ubiquitous amino acid/polyamine/organocation (APC) superfamily [5], which also includes transporters from bacteria, archaea and higher eukaryotes. These systems share a common membrane topology with 12 transmembrane segments and cytoplasmically located N- and C-terminal hydrophilic regions [6]. Transport occurs by proton symport [7,8]. Some permeases are highly specific for one

or a few related L-amino acids, whereas others have a broader specificity [2,9]. The general amino acid permease, Gap1p of *Saccharomyces cerevisiae*, transports all L- and some D-amino acids and non-protein amino acids such as citrulline and ornithine [10,11]. Ssy1 of *S. cerevisiae* does not function as a transporter but as an amino acid sensor, involved in the transcriptional regulation of the expression of other amino acid permease genes [12].

Amino acid transport has been studied most extensively in *S. cerevisiae*, in which 18 (or 24, if the more distantly related Mup1 and Mup3 (methionine permeases), Uga4 (GABA permease), Hnm1 (choline permease), Bio5 (7-keto 8-aminopelargonic acid (KAPA) permease) and Ykl174C (unknown function) are included (Ref. [2], and references therein)) members of the AAP family were identified and characterized [4,13,2]. In filamentous fungi, our current knowledge mainly relates to functional studies of amino acid transport specificity. Few of these systems have been characterized genetically and biochemically. For the β -lactam-producing filamentous fungus *Penicillium chrysogenum*, nine different amino acid transport systems have been reported on basis of their substrate specificity [14,15]. This may reflect only a minimal number as some systems may

[☆] The 1550-kb sequence containing PcMTR has been deposited in GenBank under accession number AY628328.

* Corresponding author. Tel.: +31 50 3632164; fax: +31 50 3632154.

E-mail address: a.j.m.driessen@biol.rug.nl (A.J.M. Driessen).

not be distinguished on the basis of classical transport assays only. For instance due to overlapping substrate specificity or different expression patterns, such systems might not easily be detected. Recent cloning and biochemical characterization of amino acid permeases of *P. chrysogenum* have led to the identification of the general amino acid permease PcGap1, the acidic amino acid permease PcDip5 and a permease specific for aromatic amino acids and leucine, ArLP [16,17]. In *Neurospora crassa*, five distinct transport activities (systems I–V) have been described (Ref. [1], and references therein), whereas only two genes encoding amino acid permeases have so far been characterized. Naap1 encodes a general amino acid permease [18] belonging to the AAP family, whereas Mtr functions in the uptake of neutral aliphatic and aromatic amino acids. Interestingly, the *mtr* gene was found after selection of mutants resistant to 4-methyltryptophan, a toxic tryptophan analogue. These mutants are defective in the uptake of neutral aliphatic and aromatic amino acids [19–23]. Mtr does not belong to the AAP family nor does it show significant homology with any characterized permease (less than 22% amino acid sequence identity with first hit after Blast search). However, based on its hydropathy profile, it was classified as a member of the Amino Acid/Auxin Permease (AAP) family, which includes auxin and amino acid permeases in plants and animals and vacuolar amino acid transporters in *S. cerevisiae*. The AAP family exhibits minimal sequence conservation, but they contain 11 transmembrane segments, which discriminates them from the typical 12-transmembrane segment configuration of the AAP family members [5,24]. The AAP family might represent a subfamily of the APC superfamily [4].

Here, we describe the cloning and characterization of PcMtr, an amino acid permease of *P. chrysogenum*, which is structurally and functionally homologous to Mtr of *N. crassa*.

2. Materials and methods

2.1. Strains and media

The *P. chrysogenum* strain used in this study is Wisconsin 54-1255, a low-level penicillin-producing strain (kindly provided by DSM-Anti-Infectives, Delft, The Netherlands). Cultures were started on YPG medium (1% yeast extract, 2% peptone and 2% glucose), incubated for 16 h and subsequently diluted into minimal medium supplemented with 4% glucose and 0.4% ammonium acetate as described [25]. *S. cerevisiae* M4276 strain (*MAT α* , *ura3*, *Agap1*, *Adip5*) [26] was used for the functional expression and transport assays. Cells were grown in a buffered minimal medium containing 1% succinate, 0.6% NaOH, 0.16% Yeast Nitrogen Base without ammonium sulfate and without amino acids (Difco), 2% glucose and 0.1% L-proline (MP), 0.5% (NH₄)₂SO₄ (MA) or 0.04% L-citrulline

(MC) as a nitrogen source, or on YPG. MC medium was prepared by adding filter-sterilized L-citrulline to the medium after autoclaving. Media were solidified with 1.5% agar, when needed. *E. coli* DH5 α was employed for all cloning steps, carried out as described [27].

2.2. Yeast complementation

A partial cDNA library of *P. chrysogenum* was cloned in the yeast/*E. coli* expression vector yEX-C as described [16]. *S. cerevisiae* M4276 was transformed with the plasmid DNA and spread on MC plates, containing L-citrulline as sole nitrogen source (see above). Plates were incubated at 30 °C for 5 days and colonies that appeared were transferred to fresh MC plates. Single colonies were used to inoculate liquid MA medium and, after overnight growth, plasmid DNA was isolated using a standard plasmid rescue protocol and finally transformed to *E. coli* DH5 α for propagation. The plasmid was analyzed by restriction, revealing a 1.5-kb insert of which the DNA sequence was determined. It contained an entire open reading frame of 1386 bp, which encodes a protein that shows 51% amino acid identity with the *mtr* locus encoded amino acid permease of *N. crassa* [23]. The new plasmid was designated yEX-PcMTR.

2.3. Transport assays

S. cerevisiae M4276, containing the plasmid yEX-PcMTR or the empty vector yEX-C, was used for assaying the transport characteristics of PcMtr. Transport assays were performed as described [16]. Uptake of 14 radioactively labeled amino acids (Amersham) and of ¹⁴C-L-citrulline (NEN) was measured in cells expressing PcMTR or containing the empty vector. Unless indicated otherwise, amino acids were present at a concentration of 25 μ M. Apparent *K_m* values for uptake were determined by measuring initial uptake rates (uptake after 30 s of incubation with substrate) with increasing substrate concentration within the range of 25 μ M and 2.5 mM. Apparent *K_i* values were determined in a similar manner, except that unlabeled L-tryptophan (100 μ M), L-serine (100 μ M), L-alanine (100 μ M) or L-citrulline (1 mM) was added as competitor. In competition experiments, L-phenylalanine uptake was measured at 25 μ M in the presence of a 10-fold excess of the unlabeled amino acid. All uptake measurements were made in at least two independent experiments and average values are given. The standard deviation was less than 10% at uptake levels higher than 0.5 nmol/mg protein. At lower uptake levels, variations did not exceed 30%.

2.4. Genomic search for Mtr homologs and AAP family members

Complete genome sequences of *Aspergillus nidulans*, *Neurospora crassa*, *Ustilago maydis*, *Magnaporthe grisea*,

Fusarium graminearum, *S. cerevisiae*, *Candida albicans*, *Eremothecium gossypii*, *Schizosaccharomyces pombe*, *Aspergillus fumigatus*, *Podospora anserina*, *Cryptococcus neoformans* and *Coprinus cinereus* were searched for PcMtr homologs using the BlastP and BlastX search tool [28]. Proteins with more than 50% amino acid identity with Mtr were classified as Mtr-homologs. AAP family members were searched only in protein databases of completed fungal genomes and identified on basis of homology [6]. Proteins scoring a lower E value than 10^{-25} in a Blast search [28] of the Gap1 sequence against protein databases were classified as AAP family members.

3. Results

3.1. Isolation of PcMTR

In an attempt to clone the general amino acid permease from *P. chrysogenum* [29], a cDNA library of *P. chrysogenum* was expressed in *S. cerevisiae* M4276, deficient in citrulline uptake, followed by selection on plates containing citrulline as sole nitrogen source (MC plates). In *S. cerevisiae*, citrulline can only enter the cell via Gap1, the general amino acid permease [10,30]. All colonies that appeared on MC plates after 5 to 6 days contained the same cDNA clone. Sequences revealed the presence of a gene encoding a protein which is 54% identical to the *mtr* locus encoded amino acid permease Mtr of *N. crassa* [21–23], while no significant homology was observed with AAP family permeases [3,6]. Mtr of *N. crassa* is specific for

aromatic and neutral aliphatic amino acids, but has not been reported to mediate the uptake of citrulline. The cloned permease gene was designated *PcMTR* and the encoded protein PcMtr.

Mtr is a member of the eukaryote-specific AAP family of secondary transporters. The AAP family includes amino acid and auxin permeases of plants and animals as well as vacuolar amino acid permeases of *S. cerevisiae*. Members of this sequence diverse group of transporters commonly contain 11 (or 10) transmembrane segments [5,24]. Likewise, the hydrophobicity profile of PcMtr predicts 11 transmembrane helices (data not shown).

3.2. Transport characteristics

To determine the substrate specificity of PcMtr, the uptake of 14 radioactively labeled amino acids was tested in *S. cerevisiae* M4276 expressing *PcMTR* and in control cells, containing the empty vector yex-C (Fig. 1). The amino acids were used at 25 μ M. PcMtr showed specificity for aromatic amino acids, together with the neutral, aliphatic amino acids alanine, asparagine and serine. Uptake of the basic amino acid lysine was not increased upon expression of *PcMTR*, whereas the uptake of the acidic amino acids glutamate and aspartate was only slightly increased. This indicates that charged amino acids are poor (or not at all) substrates for PcMtr. The reduced level of proline uptake in cells overexpressing *PcMTR* is unclear, but this might relate to a reduced expression of endogenous proline permease(s) upon the overexpression of *PcMTR* while cells are grown on a medium with proline as nitrogen source. Therefore, we

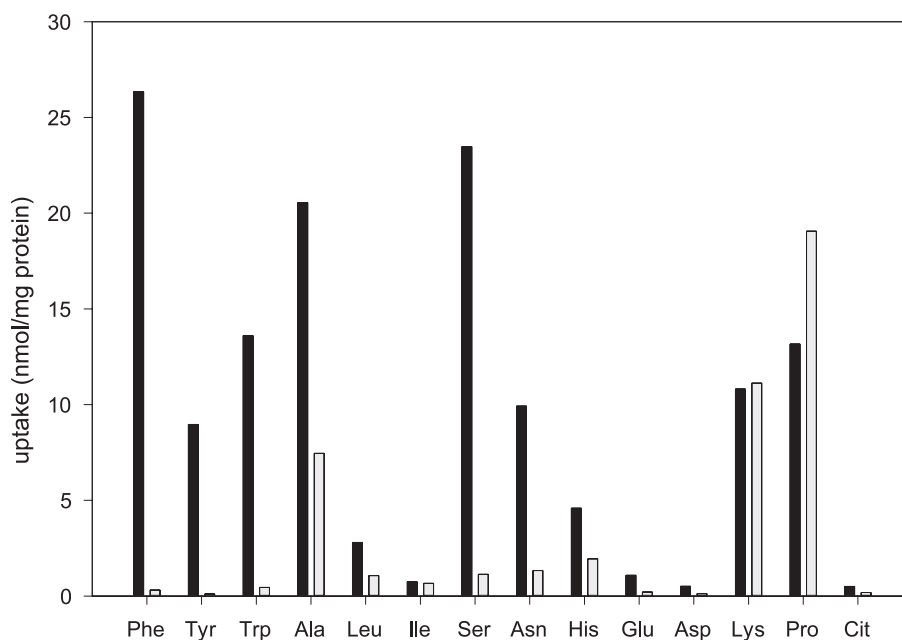


Fig. 1. Uptake of amino acids in *S. cerevisiae* M4276 expressing *PcMTR* (solid bars) or in control cells containing the empty vector (grey bars). Uptake experiments were performed with cells grown to exponential phase on minimal proline medium. Expression of *PcMTR* was induced with 0.2 mM CuSO_4 , 5 h before harvesting. The ^{14}C -labeled L-amino acids were present at 25 μ M concentration and the uptake levels were determined after 3-min incubation. The results shown are the means of three independent experiments.

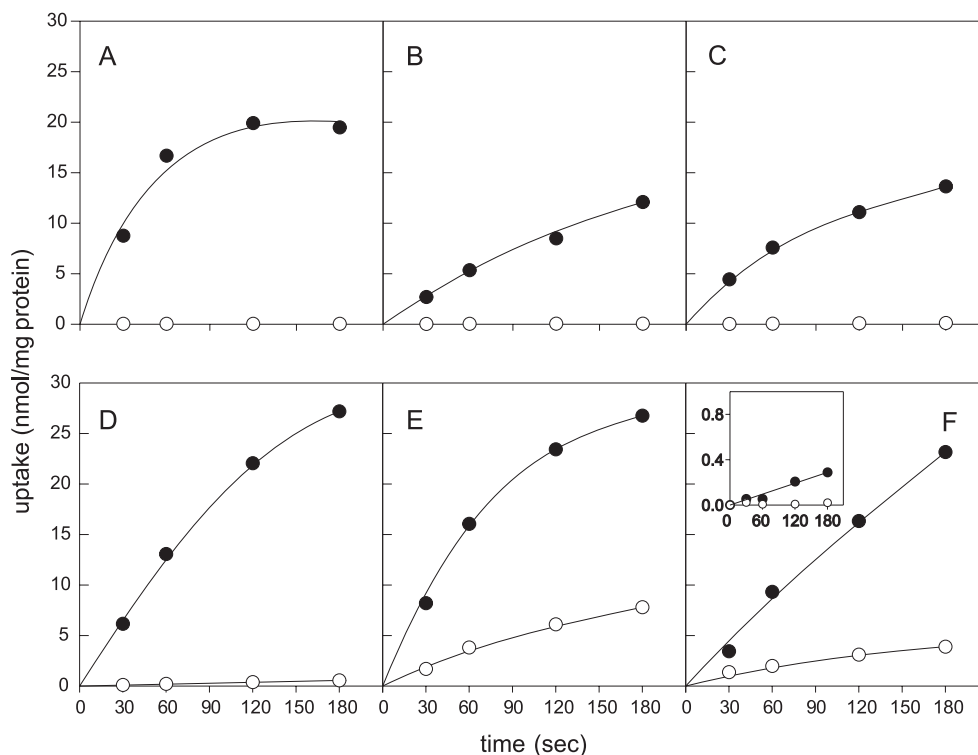


Fig. 2. Time-dependent uptake of neutral and aromatic amino acids in *S. cerevisiae* M4276 cells expressing *PcMTR* (solid circles) or in control cells containing the empty vector (open circles). (A) L-Phenylalanine, (B) L-tyrosine, (C) L-tryptophan, (D) L-serine, (E) L-alanine, (F) L-citrulline. ^{14}C -labeled L-amino acids were present at 25 μM concentration, except for L-citrulline uptake, which was measured at 2.5 mM (F) or at 25 μM concentration (F, inset). Details are as described in the legend to Fig. 1 and Materials and methods.

cannot determine if *PcMTR* catalyzes the uptake of proline. Surprisingly, uptake of L-citrulline at 25 μM by *PcMTR* expressing cells was relatively low as compared to the other substrates. Citrulline uptake was also measured at 2.5 mM,

which is close to the concentration of 2.3 mM used in the plates during the selection procedure of *S. cerevisiae* M4276 transformants (Fig. 2, last panel). Herein, the uptake by cells expressing *PcMTR* was 2 orders of magnitude higher than at

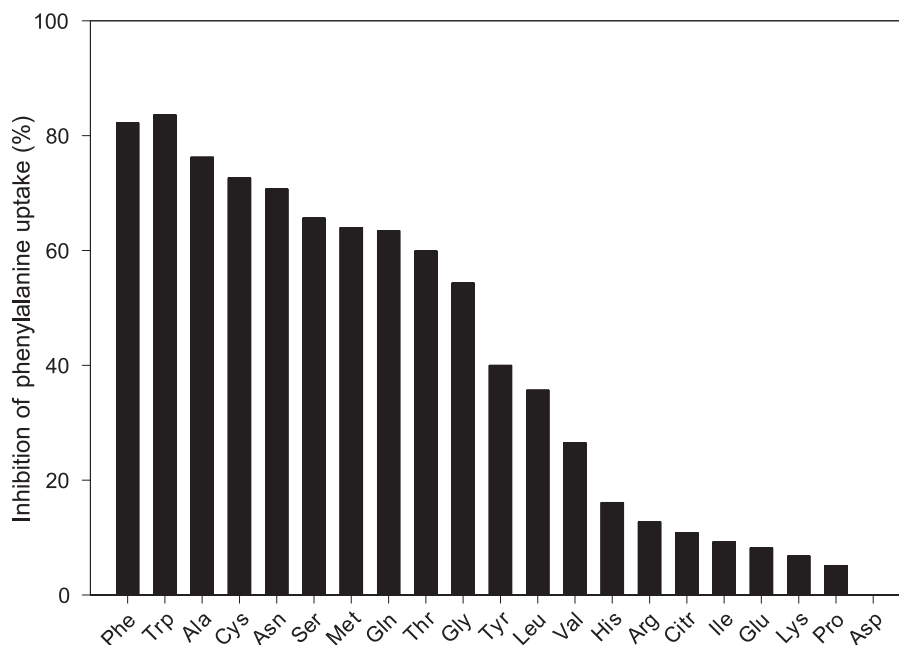


Fig. 3. Substrate specificity of *PcMtr* as scored by the effect of an excess of unlabeled amino acids on the uptake of ^{14}C -L-phenylalanine by *S. cerevisiae* strain M4276 expressing *PcMTR*. L-Phenylalanine was present at a final concentration of 25 μM , and the indicated unlabeled amino acids were present in a 10-fold excess. The uptake was determined after 3 min and the results shown are the means of three independent experiments.

25 μM . This shows that PcMtr has a low-affinity for citrulline, but is capable of transporting citrulline with high capacity into the cell, which explains why these cells can grow on citrulline as a sole nitrogen source. The substrate specificity of PcMtr corresponds with that reported for Mtr of *N. crassa* [20,19], with phenylalanine and tryptophan as preferred substrates (Fig. 2).

To further characterize the substrate specificity of PcMtr, the inhibitory effect of a 10-fold excess of unlabeled amino acids on the uptake of ^{14}C -L-phenylalanine via PcMtr was measured (Fig. 3). Strikingly, tyrosine that differs from phenylalanine by the presence of a hydroxyl group in the benzyl-side chain is a relatively poor competitor as compared to most neutral amphipathic amino acids. The affinity (K_m) for aromatic amino acid transport was determined, as well as the inhibitory constants (K_i) on phenylalanine transport (Table 1). Because of high background level of alanine uptake by endogenous permeases and the apparently very low affinity of PcMtr for citrulline (K_i on phenylalanine transport >10 mM), it was not possible to determine the K_m for uptake of these substrates. Except for the presence of a neutral side chain, amino acid substrates of PcMtr do not share any common features. Substrates include large and small (phenylalanine and alanine), and hydrophilic or hydrophobic (serine and tryptophan) amino acids, and the transport affinity is in the range of 30 to 150 μM (Table 1). As uptake measurements were done in a high affinity mode, amino acids, apart from citrulline, that are possibly transported, but with low affinity, may not have been identified as such.

3.3. Distribution of Mtr and AAP type permeases in fungi

To determine the abundance of Mtr type of transporters in fungi, completed fungal genome sequences were scored for the presence of AAP and Mtr type of transporters. Using the BLAST search tool (BlastX or BlastP [28]), 5 of the 12 fungal genomes contained a single Mtr homolog (more than 50% amino acid identity). Remarkably, *F. graminearum* contains five homologs, and the similarity among these

Table 1
Affinity (K_m) and inhibitory (K_i) constants for PcMtr-mediated amino acid uptake

Amino acid	Kinetic parameter	
	K_m (μM)	K_i (μM) ^a
Phenylalanine	28	–
Tyrosine	200	n.d.
Tryptophan	42	38
Serine	n.d.	110
Alanine	n.d.	130
Citrulline	n.d.	$>10,000$

n.d., not determined.

^a K_i values were estimated from the inhibitory effect of the indicated amino acids on the uptake of L-phenylalanine as described in Materials and methods.

Table 2

Distribution of Mtr homologs and AAP type permeases in fungal genomes

		Number of transporters		Genome size (Mb)
		Mtr homologs	AAP members	
Ascomycetes	<i>A. nidulans</i>	1	20–23	31
	<i>F. graminearum</i>	5	23	40
	<i>A. fumigatus</i>	1	n.d. ^a	32
	<i>P. anserina</i>	1	n.d.	34
	<i>N. crassa</i>	1	9	40
	<i>M. grisea</i>	1	14	40
	<i>S. cerevisiae</i>	0	18	12
	<i>E. gossypii</i>	0	15	9.2
	<i>C. albicans</i>	0	18–20	16
basidiomycetes	<i>S. pombe</i>	0	12	14
	<i>U. maydis</i>	1	8	20
	<i>C. cinereus</i>	0	n.d.	38
	<i>C. neoformans</i>	0	n.d.	20

Genome databases were obtained from NCBI (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=euk), except for the *P. anserina* genome, which was obtained from the *P. anserina* Genome Project, Institut de Génétique et Microbiologie, Université de Paris-Sud (<http://podospora.igmors.u-psud.fr/index.html>), and the *A. fumigatus* genome, which was obtained from the *A. fumigatus* Genome Database of The Institute for Genomic Research (TIGR, <http://www.tigr.org/tdb/e2k1/afu1>).

^a n.d., not determined. The genomes are not annotated.

sequences varies from 51% to 60% amino acid sequence identity. The yeasts *S. cerevisiae*, *C. albicans*, and *E. gossypii*, the fission yeast *S. pombe*, and the basidiomycetes

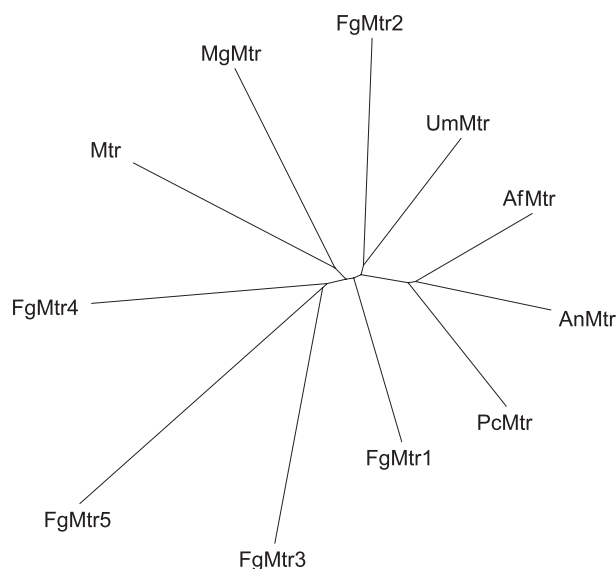


Fig. 4. Phylogenetic relationship between fungal Mtr homologs. Mtr homologs were identified in the completed fungal genomes using BlastP and the *N. crassa* Mtr (accession number EAA33125) as a probe: *F. graminearum* (FgMtr 1–5, accession numbers EAA78089, EAA78322, EAA72970, EAA71441 and EAA76673, respectively); *M. grisea* (MgMtr, accession number EAA56571); *U. maydis* (UmMtr, accession number EAK81441); *A. fumigatus* (AfMtr, deduced by BlastX against the *A. fumigatus* database (TIGR) and in silico analysis of DNA sequence of contig 5172); *A. nidulans* (AnMtr, accession number EAA58464). The alignment and tree were created using ClustalX and the neighbor joining method, version 3.6 from the PHYLIP package (Joe Felsenstein, Department of Genome Sciences, University of Washington).

C. neoformans and *C. cinereus*, do not contain an Mtr homolog (Table 2). An alignment and phylogenetic tree of Mtr homologs show that the proteins of *P. chrysogenum*, *A. nidulans* and *A. fumigatus* cluster together (Fig. 4). The divergence among Mtr homologs does not follow the phylogenetic evolutionary tree as PcMtr is more homologous to the Mtr homolog of the basidiomycete *U. maydis* than of the ascomycete *N. crassa*. Also, the five Mtr homologs of *F. graminearum* do not cluster together.

In addition, the number of AAP family members was determined in the various fungal genomes using BlastP. In all cases except *A. nidulans*, a clearly distinguishable group of proteins shows significant homology with established AAP family members (E values lower than 10^{-25}). Their number ranges from eight in the basidiomycete *U. maydis* to more than 18 in *A. nidulans*, *F. graminearum*, *S. cerevisiae* and *C. albicans* (Table 2). This indicates that in many fungi, AAP-like transporters represent the major class of transporters while Mtr type amino acid permeases are rare and not universally present in fungal genomes.

4. Discussion

Here, we describe the cloning and characterization of PcMtr, an amino acid permease of *P. chrysogenum* that exhibits specificity for aromatic and neutral aliphatic amino acids. Complementation of citrulline uptake in *S. cerevisiae* M4276 yielded only PcMtr, and not the expected general amino acid permease (or Gap). Nevertheless, the *P. chrysogenum* Gap was recently shown to transport citrulline [16], implying that unlike in *S. cerevisiae*, multiple pathways exist in *P. chrysogenum* for the uptake of citrulline. PcMtr has a relatively broad specificity, as substrates include hydrophobic and hydrophilic as well as bulky and small residues. Amino acids with a charged side chain are not transported.

PcMtr and the functionally and structurally homologous Mtr transporter of *N. crassa* [22,23] are not related to the abundant family of AAP transporters [3], a subfamily of the APC family [4]. Instead, they represent a small class of their own which can be subdivided in the AAAP family that includes auxin and amino acid permeases in plants and animals and vacuolar amino acid transporters in *S. cerevisiae* [5]. The homology of the amino acid sequences of PcMtr and its homologs with other AAAP family members is low (less than 25% identity), but they share the common hydropathy profile that suggests the presence of 11 transmembrane segments [5]. Filamentous fungi appear to contain only a single gene that specifies an Mtr type transporter, although *F. graminearum* is exceptional in that it contains five homologs. However, Mtr-like transporters are lacking in some yeast and filamentous fungi. None of these systems has been studied but considering the fact that the AAP-like amino acid transporters are relatively abundant in filamentous fungi (8–20 different systems), it seems likely that the

Mtr type transporters are involved in a specific function such as the uptake of a signaling molecule. This would provide a possible explanation that the wheat pathogen *F. graminearum* contains five Mtr homologs. In particular, expression analysis of these transporters may shed more light on their possible physiological function and significance.

Acknowledgments

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