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Published in:
Biochimica et Biophysica Acta-Biomembranes

DOI:
10.1016/S0005-2736(02)00510-2

Publication date:
2002

ELSEVIER

Biochimica et Biophysica Acta 1565 (2002) 73–80

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Cloning and characterization of an aromatic amino acid and leucine permease of *Penicillium chrysogenum*

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Received 26 March 2002; accepted 10 July 2002

Abstract

The gene encoding the amino acid permease ArlP (*Ar*omatic and *l*eucine *P*ermease) was isolated from the filamentous fungus *Penicillium chrysogenum* after PCR using degenerated oligonucleotides based on conserved regions of fungal amino acid permeases. The cDNA clone was used for expression of the permease in *Saccharomyces cerevisiae* M4054, which is defective in the general amino acid permease Gap1. Upon overexpression, an increase in the uptake of L-tyrosine, L-phenylalanine, L-tryptophan and L-leucine was observed. Further competition experiments indicate that ArlP recognizes neutral and aromatic amino acids with an unbranched β-carbon atom.

Keywords: Amino acid permease; *Penicillium chrysogenum*; Gene expression; *Saccharomyces cerevisiae*; Amino acid uptake

1. Introduction

Fungi can use amino acids either as nitrogen and/or carbon source or as building blocks for protein synthesis. The uptake of amino acids is mediated by a multiplicity of carriers with different, overlapping degree of specificity. In addition, cells harbor one or more broadly specific, large capacity, general amino acid permeases [1,2]. Most of the sequenced and characterized fungal amino acid permeases belong to one related group, referred to as the AAP family [3]. This family also includes some bacterial amino acid permeases. These transport systems have a common structural organization with 12 putative α-helical transmembrane segments and cytoplasmically located N- and C-terminal hydrophilic regions [2]. Transport occurs by proton symport with the pmf as driving force [4,5].

In *Neurospora crassa*, an amino acid permease specific for neutral aliphatic and aromatic amino acids was identified, which is not related to the AAP family [6–8]. It is a member of the amino acid/auxin permease (AAAP) family, which includes auxin and amino acid permeases from plants and animals [9].

In the yeast *Saccharomyces cerevisiae*, 24 members of the AAP family have been found [3,10,11] of which most have been functionally characterized [12]. Based on their regulation at the transcriptional level, the permeases in *S. cerevisiae* are divided in two classes. The general amino acid permease Gap1p, which transports all D- and L-amino acids with high capacity [13], the proline permease Put4p [14] and the acidic amino acid permease Dip5p [15] are subject to nitrogen catabolite repression. The transcription of their genes is repressed when a rich nitrogen source like ammonia or arginine is available [16]. The expression of most low-capacity, highly specific and high-affinity permeases requires induction by sensing of an extracellular amino acid [17]. Examples are the branched-chain amino acid permeases, Bap2p [18] and Bap3p, the tyrosine and tryptophan permease Tat1p [19] and the histidine permease, Hip1p [20].

In filamentous fungi, amino acid uptake has been studied mainly by transport assays and characterization of mutants defective in uptake of one or more amino acids. In *N. crassa,* five distinct transport systems (systems I–V) have been identified. System III corresponds to the general amino acid permease. The other systems are specific for a group of related amino acids. A similar situation seems to hold for *Aspergillus nidulans* (Ref. [1] and references therein). The L-proline specific transport system, encoded by *prnB*, was

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PII: S0005-2736(02)00510-2

the first amino acid permease from a filamentous fungus that has been sequenced [21] and characterized with respect to kinetics and regulation [22]. For the penicillin-producing filamentous fungus *Penicillium chrysogenum*, nine different transport systems have been reported: system I for L-methionine [23]; II for L-cysteine [24]; III for neutral and basic amino acids [25]; IV for acidic amino acids; V for L-proline; VI for L-lysine and L-arginine, VII for L-arginine; VIII for L-lysine and IX for L-cysteine [26]. The first two systems are expressed under sulfur starvation, while systems III–V are expressed under nitrogen and carbon starvation (NCR and CCR). Systems VI–VIII appear constitutively [1]. Here we describe the cloning of a gene that specifies a novel amino acid permease from *P. chrysogenum* with an unusual specificity.

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). Cultures were started on YPG medium (1% yeast extract, 2% peptone and 2% glucose) and incubated for 16 h and subsequently diluted into minimal medium supplemented with 4% glucose and 0.4% ammonium acetate as described by Ref. [27]. *S. cerevisiae* M4054 strain (*MATα*, *ura3*, *Δgap1*) [18] 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% proline as a nitrogen source (MP), or on YPg. Minimal medium containing 0.5% $(NH_4)_2SO_4$ instead of proline was used for selection of transformants containing the plasmid yCA and yCN (see below). *E. coli* DH5α was employed for all cloning steps, carried out as described in Ref. [28].

2.2. Amplification of amino acid permease gene fragment by PCR

Degenerated oligonucleotides (5′-ATGATSKCCMTSGGYGSYISYMTIGGIACIGG-3′ and 5′-GAAYTCGRIYTCRSCRWAIGGICG-3′) were designed based on conserved regions of fungal amino acid permease genes. PCR was performed on chromosomal DNA of *P. chrysogenum* Wisconsin 54–1255 in a Perkin-Elmer thermocycler for 30 cycles, with each cycle consisting of 94 °C for 45 s, 40 °C for 1 min and 72 °C for 1 min. At the end of 30 cycles, samples were incubated for 5 min at 72 °C. Gel purified products, ranging from approximately 350 to 500 bp were subcloned into the pGEM-T Easy vector (Promega) for DNA sequence determination. Of the 25 clones sequenced, two identical clones showed homology with known amino acid transporter-encoding genes.

2.3. Isolation of permease gene from genomic library

A genomic library of *P. chrysogenum* DNA in phage Lambda ZAP II (Stratagene), was screened by plaque hybridization as described by Ref. [28]. Approximately 30,000 plaques were transferred to Zeta-Probe blotting membranes (BioRad), and probed with the radioactively labeled above-mentioned PCR product. One positive plaque was isolated, phages were released and pBluescript was rescued from the Lambda ZAP II vector by in vivo excision, using M13. The 8-kb insert carried the complete permease gene.

2.4. Expression in S. cerevisiae M4054

For expression, a construct was created based on the yeast–*E. coli* shuttle vector yEP352 [29]. The copper inducible promoter pCUP1 of *S. cerevisiae* was inserted into yEP352. The cDNA of the amino acid permease gene was amplified by PCR from a cDNA library and cloned behind pCUP1. The terminator of *AatA*, encoding acyl coenzyme A:isopenicillin N acyltransferase of *P. chrysogenum*, was cloned behind the permease gene resulting in the plasmid yCA. For control experiments, the same construct without the permease gene was used, denoted yCN. Plasmids were transformed into *S. cerevisiae* strain M4054. To test transcript levels, total RNA was isolated from cells disrupted by vortexing with glassbeads and extracted with Trizol reagent (Gibco BRL). Specific *arlP* mRNA levels were measured by quantitative RT-PCR.

2.5. Transport studies

Overnight cultures grown in minimal medium containing proline as the sole nitrogen source were diluted with fresh medium, and growth was continued at 30 °C until an OD_{600} of 0.4–0.8. Cells from 50 ml of culture were harvested, washed in minimal medium without any nitrogen source and resuspended to OD_{600} of 10 in the same medium. After 10 min of preincubation at 30 °C, 100 μl of cells were added to 150 μl of the same medium containing 25 μM ^{14}C-labeled L-amino acid (0.05 μCi). For competition studies, the uptake of 250 μM [^{14}C-] L-tyrosine was analyzed in the presence of a 10-fold excess of an unlabeled L-amino acid. For the determination of the K_m value, the concentration of L-tyrosine was varied between 25 μM and 2.5 mM, while for K_I determinations, the kinetics was analyzed in the presence of 500 μM of the indicated unlabeled amino acid. The uptake of amino acid was stopped by the addition of 2 ml of ice-cold 0.1 M LiCl, immediately followed by filtration through a 0.45-μm pore size nitrocellulose filter. The filters were washed with 2 ml of ice-cold 0.1 M LiCl, submerged in scintillation fluid and the retained radioactivity was measured in a scintillation counter.

2.6. Expression of arlP

For expression studies, main cultures were incubated for 48 h (early stationary phase) in a rotary shaker at 200 rpm and 25 °C, then transferred to fresh minimal medium containing either urea (0.4%, w/v), ammonium acetate (0.4%, w/v) or ammonium acetate plus 10 mM tyrosine, phenylalanine or leucine as nitrogen source. Incubation was continued for 5 h, and mycelial samples were taken at various times, washed and ground with liquid nitrogen. RNA was extracted using Trizol reagent (Gibco BRL) and specific *arlP* mRNA levels were measured by quantitative RT-PCR.

3. Results

3.1. Cloning of amino acid permeases of P. chrysogenum

To clone genes encoding the *P. chrysogenum* amino acid permeases, PCR was performed using degenerated primers based on conserved regions of fungal amino acid permeases (Fig. 1). Products that varied in length from about 350 to 500 bp were cloned, sequenced and analyzed by means of BLAST searches [30]. Of the 20 different PCR products, only one fragment of 406 bp showed significant homology (up to 37% amino acid identity) with genes encoding amino acid permeases of *S. cerevisiae* and other fungi. To clone the complete gene, a genomic library of *P. chrysogenum* in lambda ZAP II (Stratagene) was screened by plaque hybridization. One positive plaque was isolated, and by in vivo excision from the lambda ZAP II phage vector, pBluescript, containing an insert of 8 kb, was obtained. The insert was sequenced and found to contain an ORF that specifies a permease gene. The full-length sequence of *arlP* is shown in Fig. 2. The ORF of 1680 bp is interrupted by one intron of 59 bp. The encoded transport protein shows the highest homology with putative amino acid transporters of *Uromyces fabae* (41% amino acid identity), *Amanita muscaria* (39%), *Schizosaccharomyces pombe* (39%) and with characterized amino acid transporters of *S. cerevisiae* (Dip5, 37%; Put4, 36%) (BLAST search [30]).

3.2. Expression in S. cerevisiae M4054

The gene was expressed in *S. cerevisiae* strain M4054 (*Δgap1*) to analyze the substrate specificity of the permease. This strain lacks the general amino acid permease, Gap1. Gap1 is a nonspecific, high-capacity permease that transports most L- and D-amino acids. Due to the deletion, strain M4054 exhibits a low endogenous amino acid uptake activity [12,13]. The permease gene was amplified from a cDNA library, thereby obtaining the intron-less ORF, and inserted into the yEP352-based vector yCN, under control of the copper inducible *CUP1* promoter, yielding yCA. This construct was introduced into strain M4054. For control experiments, the "empty vector" yCN was transformed into M4054. To determine if the gene was expressed upon addition of $CuSO_4$, mRNA levels were analyzed by RT-PCR. The presence of $CuSO_4$ resulted in a high transcription of the permease gene, whereas without $CuSO_4$, only a low level of transcript was found (see also Fig. 5).

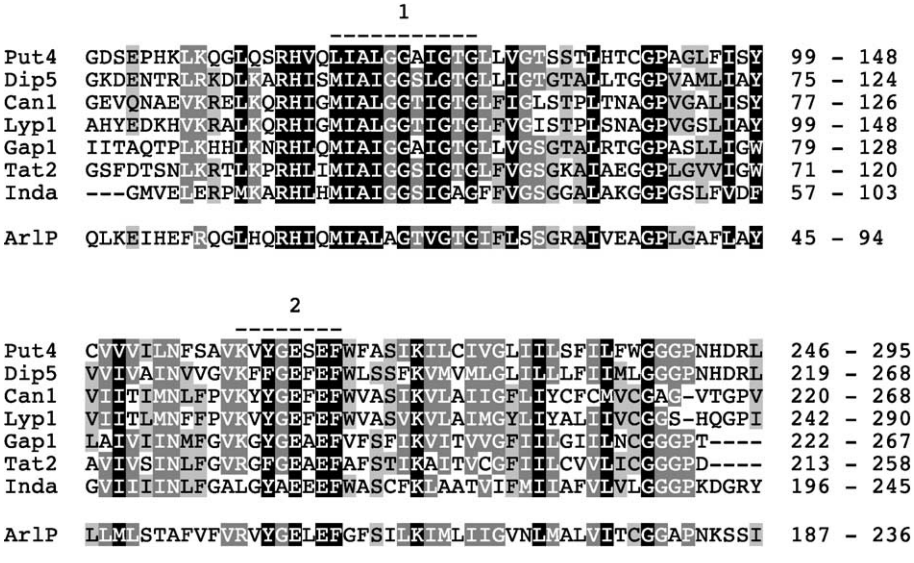

Fig. 1. Amino acid sequence alignment of conserved regions of fungal amino acid permeases. Sequences were searched from PubMed database at NCBI (http://www.ncbi.nlm.nih.gov/PubMed) using the program BLAST [30], except for the ArlP sequence (this work). Alignments were made with ClustalX [36]. Degenerated oligonucleotides were based on the indicated sequences (dashed lines). Put4 [37], Dip5 [15], Can1 [38], Lyp1 [39], Gap1 [13] and Tat2 [19] are *S. cerevisiae* permeases for proline, glutamate and aspartate, arginine, all amino acids and aromatic amino acids, respectively; Inda is a general amino acid permease from *T. harzianum* [40].

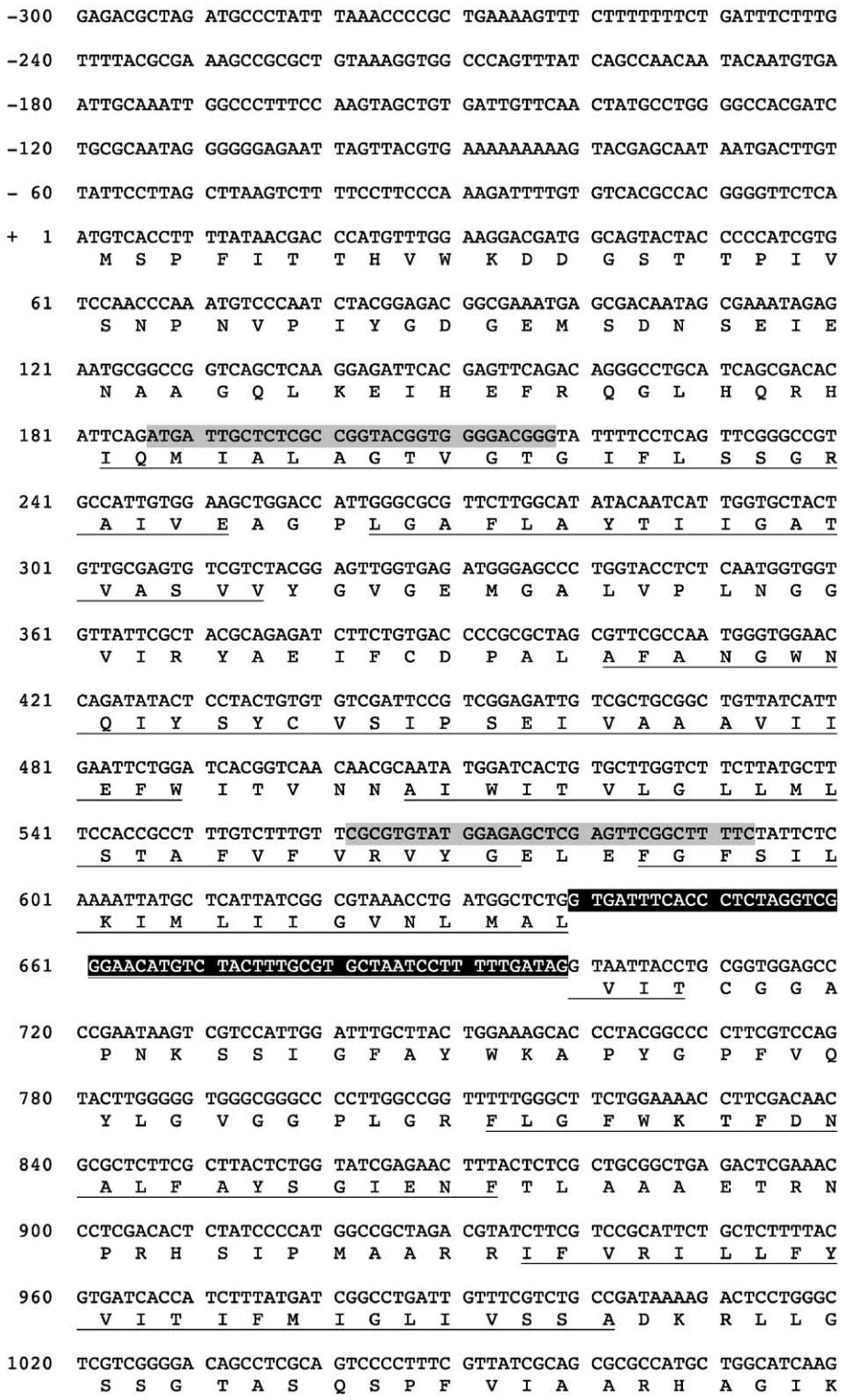

Fig. 2. Nucleotide sequence and deduced amino acid sequence of the genomic clone of *P. chrysogenum* ArlP. Sequences with gray background indicate the position of the conserved regions of AAP family permeases, on which degenerated primers were based. The 59 bp intron is boxed in black. The 12 predicted transmembrane segments are underlined.

```
1080  GTGGTTCCTT CGATCATCAA CGCAGTTGTG CTCACATCAG CATGGTCCTC CGGGAACAGC
        V  V  P   S  I  I   N  A  V  V   L  T  S   A  W  S   S  G  N  S

1140  AATATCTTGG GTGGCTCCAG AATCCTATAC GGTATGGCAA CCCAGGGTCA TGCCCCCGCC
        N  I  L   G  G  S   R  I  L  Y   G  M  A   T  Q  G   H  A  P  A

1200  GTCTTCACAC GTATCAACCG CTTCGGTATC CCTTGGGTTG CTGTCGCGCT CTATGGCGTC
        V  F  T   R  I  N   R  F  G  I   P  W  V   A  V  A   L  Y  G  V

1260  TTCATGTCAC TTGGATACAT GAGTCTTTCC AGCTCGGCCA GCACGGTATT CACATGGCTA
        F  M  S   L  G  Y   M  S  L  S   S  S  A   S  T  V   F  T  W  L

1320  CAGAACCTTG TCTCTATCTC GACTCTAGTG AACCTGATGT GCATCTGTAT TGTCTACCTT
        Q  N  L   V  S  I   S  T  L  V   N  L  M   C  I  C   I  V  Y  L

1380  AGATTCTACT ACGGATGCAA GAAGCAGGGA ATTGATCGCT TCAAGGAGCT CCCGTGGGCA
        R  F  Y   Y  G  C   K  K  Q  G   I  D  R   F  K  E   L  P  W  A

1440  GCACCTTTCC AGCCATACAT AACTTGGATC TCGTTGTTCA TTTATGTTGT CCTTTTCTTC
        A  P  F   Q  P  Y   I  T  W  I   S  L  F   I  Y  V   V  L  F  F

1500  ACCGGAGGGT TTACGACATT TATGCGCGGT CATTGGAGCA CTGCAACCTT CGTTTCGACT
        T  G  G   F  T  T   F  M  R  G   H  W  S   T  A  T   F  V  S  T

1560  TATTTCAACC TGCCATTCAT CGTCATTGTG TATTTTGCCT ACAAGTTCTG GGCAAAAACG
        Y  F  N   L  P  F   I  V  I  V   Y  F  A   Y  K  F   W  A  K  T

1620  AAGATCATCC CGCTGGCAGA AATTCCCATT CGGCCTTTCA TCGAAAGCTG GCACAAAAAT
        K  I  I   P  L  A   E  I  P  I   R  P  F   I  E  S   W  H  K  N

1680  CCCGAGCCAG AGCCCAAGCC GAAGCGAGGC CTCAGCAAAC TTAATATTTT GTGGTCATAA
        P  E  P   E  P  K   P  K  R  G   L  S  K   L  N  I   L  W  S  -

1740  TTTGTACCGT GTACAGTTTC ACCATGAACC TGACGCTGGA CCACCGGACA CCGGCCTGTC

1800  CGGTTTTAGG GCTTAGTGGC CGGGTGATGC CGGGCCCGCT TTCATGCATC GAAAACCGTG

1860  ACATTGTGCT TGCTGACACT AAATAAAAAG TTTCTTTGCT TCATTATATT TGAGAATACT
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Fig. 2 (*continued*).

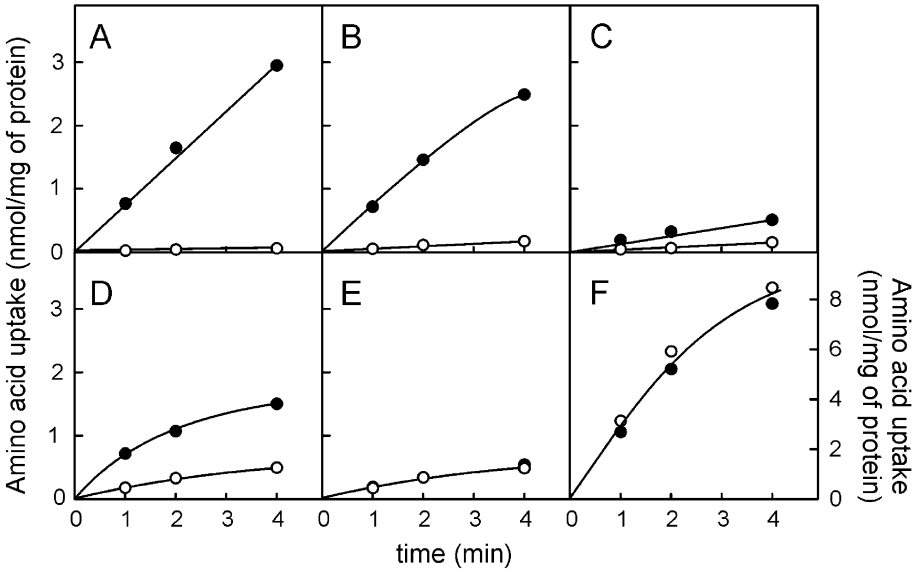

Fig. 3. Time-dependent uptake of L-tyrosine (A), L-phenylalanine (B), L-tryptophan (C), L-leucine (D), L-isoleucine (E) and L-alanine (F) in *S. cerevisiae* M4054 expressing ArlP (solid symbols) or with empty vector (open symbols). Uptake experiments were performed with cells grown to exponential phase in minimal proline medium. Expression of ArlP was induced with 2 mM $CuSO_4$ 5 h before harvesting.

3.3. Transport characteristics

To determine the substrate specificity of the permease gene expressed in *S. cerevisiae* M4054, uptake was analyzed of 15 different [^{14}C]-radiolabeled L-amino acids that represent all groups of amino acids. Cells were grown to mid-exponential phase in minimal medium with proline as sole nitrogen source to prevent nitrogen catabolite inhibition known to elicit the degradation of some amino-acid permeases in *S. cerevisiae* [31]. The uptake of L-[^{14}C]tyrosine in M4054 expressing the permease was at least 10 times higher than the background uptake level (Fig. 3). A significant increase in the uptake rate was also observed for L-[^{14}C]phenylalanine, L-[^{14}C]leucine and L-[^{14}C]tryptophan. For the other tested amino acids (L-[^{14}C]alanine, L-[^{14}C]isoleucine, L-[^{14}C]lysine, L-[^{14}C]glutamine, L-[^{14}C]asparagine, L-[^{14}C]cysteine, L-[^{14}C]glutamate, L-[^{14}C]histidine, L-[^{14}C]threonine, L-[^{14}C]serine and L-[^{14}C]proline), the uptake rates were indistinguishable for the control and permease-containing cells.

The apparent K_m of tyrosine uptake was about 360 μM (Table 1). The substrate specificity of the permease was further tested by determining the inhibition of uptake of L-[^{14}C]tyrosine by a 10-fold excess of unlabeled amino acids (Fig. 4). Interestingly, some of the amino acids that inhibited tyrosine uptake by more than 60%, such as alanine, arginine and methionine, appeared not to be transported by the permease in direct transport assays. Leucine, tryptophan and phenylalanine were strong competitive inhibitors of L-[^{14}C]tyrosine with K_i values ranging from 150 to 750 μM (Table 1). None of the amino acids that contained a branched β-carbon (i.e. isoleucine, valine and threonine) inhibited tyrosine uptake. The acidic amino acids exerted low inhibition, which might be related to their negative charge. Based on these data, the transporter that is now termed ArlP appears to be involved in the uptake of aromatic amino acids and leucine.

The activity of the permease upon Cu^{2+}-induced overexpression of *arlP* in *S. cerevisiae* was tested not only after growing cells in minimal medium with proline as sole nitrogen source but also with NH_4^+. Uptake of tyrosine in cells grown in the presence of $(NH_4)_2SO_4$ was much lower than in cells grown with proline as sole N-source. Since the transcript levels of the *arlP* gene were not drastically different with both nitrogen sources, this suggests a negative regulation of the amino acid permease at a posttranscriptional level in response to ammonia (Fig. 5). Posttranscriptional regulation by rapid ammonia inactivation has been shown for the proline permease PrnB in *A. nidulans* [22], and for Gap1 in *S. cerevisiae* [32].

Table 1
Kinetic parameters for tyrosine uptake and competitive inhibition of tyrosine uptake by phenylalanine, tryptophan and leucine

Amino acid	K_m (μM)	K_1 (μM)
Tyrosine	361	
Phenylalanine		355
Tryptophan		743
Leucine		169

The values were obtained from linear regression analysis of data in Lineweaver–Burk plots for competitive inhibition.

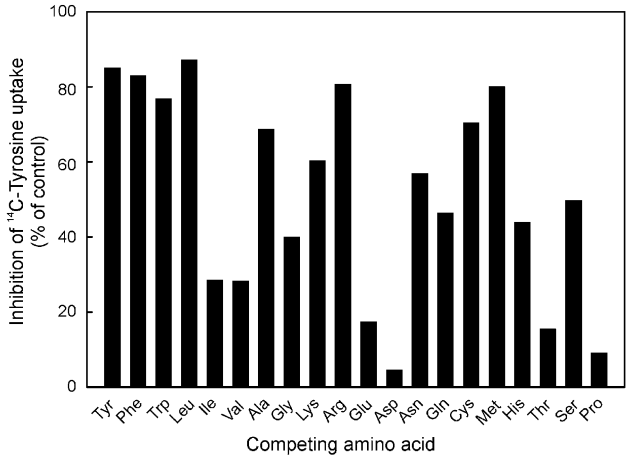

Fig. 4. Competition of unlabeled amino acids for uptake of [^{14}C]tyrosine by *S. cerevisiae* strain M4054 expressing ArlP. [^{14}C]tyrosine was present at a final concentration of 25 μM, and the indicated unlabeled amino acids were added in a 10-fold excess. The rate of uptake was determined after 3 min and results shown are the means of three independent experiments.

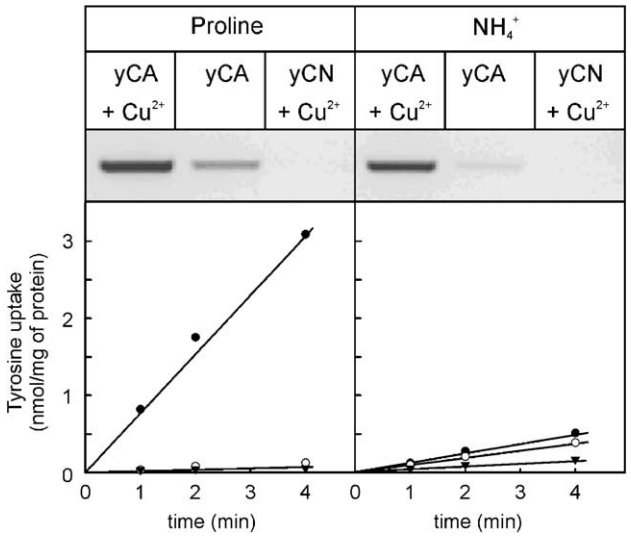

Fig. 5. Expression (upper panel) and tyrosine uptake activity (lower panel) of ArlP in *S. cerevisiae* strain M4054 transformed with yCA (containing *arlP*) or yCN (empty vector) grown in the presence of Cu^{2+} or without Cu^{2+}, in minimal proline medium (MP, left) or minimal ammonia medium (MA, right). *ArlP* mRNA levels were measured by a quantitative RT-PCR. Tyrosine uptake was measured at an extracellular concentration of 25 μM in minimal medium without nitrogen source (MM). ArlP mediated uptake of tyrosine was measured in cells grown in MA (with Cu^{2+}; closed circles; without Cu^{2+}; open circles) and MP (Cu^{2+}; closed circles).

3.4. Expression of arlP in P. chrysogenum

The two most common mechanisms involved in regulation at the transcriptional level of AAP family transporters are substrate induction [33] and nitrogen catabolite repression [3]. To test if the expression of *arlP* is regulated by either one of these mechanisms, *P. chrysogenum* was grown in minimal media with proline (poor nitrogen source) or ammoniaoxaloacetate (rich nitrogen source) as the sole nitrogen source, as well as in presence of millimolar concentrations of tyrosine, phenylalanine or leucine. Messenger RNA levels of the permease gene were measured by RT-PCR. At none of the tested conditions, a significant level of expression could be detected during logarithmic growth, while low expression levels were found at late stationary phase, irrespective of carbon or nitrogen source.

4. Discussion

In order to amplify gene fragments of amino acid permeases of *P. chrysogenum*, PCR was performed using a set of degenerated primers that were based on conserved regions of fungal amino acid permeases, forming the AAP family [2,3]. The PCR fragment was used to clone the entire gene from a genomic library. The cloned permease gene showed 30–39% amino acid sequence identity with members of this family. When the cDNA clone was expressed in *S. cerevisiae* strain M4054 (*Δgap1*), which has a decreased amino acid uptake [18], a significantly increased rate of tyrosine, phenylalanine, leucine and tryptophan uptake was observed. For other amino acids, the increase in uptake was only marginal. Based on this observation, the permease was identified as an aromatic amino acid and leucine transport system, termed ArlP.

An interesting feature of ArlP is its selectivity towards the configuration of the β-carbon of amino acids. When the side chain is branched at this position, the amino acid does not compete with tyrosine for uptake. This suggests that the active site has an unusual specificity that has not been observed before for aromatic amino acid permeases. However, many of the competing amino acids are not transported by ArlP as the uptake of these amino acids is not elevated upon expression of *arlP* in *S. cerevisiae* M4054.

The physiological function of ArlP is not understood. The expression of the *arlP* gene was tested under conditions of nitrogen limitation/starvation and in the presence of millimolar concentrations of its substrates. No significant level of the *arlP* mRNA could be detected under these conditions. Some expression was observed at late stationary phase, irrespective of carbon or nitrogen source, but this was not further analyzed. Some fungal amino acid permeases have been shown to be expressed in differentiating cells. For instance, the expression of an amino acid permease of the rust fungus *U. fabae*, which is homologous to the amino acid permeases of the AAP family, is induced only in haustoria, i.e. specialized hyphae that are involved in plant infection [34]. The expression of *arlP* in *P. chrysogenum* may also be restricted to a morphologically or developmentally differentiated state.

We also noted that ArlP activity after overexpression was strongly decreased in *S. cerevisiae* when grown with ammonia as nitrogen source. Posttranscriptional regulation by rapid ammonia inactivation has been shown for the proline permease PrnB in *A. nidulans* [22], and in more detail, for Gap1 in *S. cerevisiae,* where NH_4^+ induces ubiquitination, endocytosis and vacuolar degradation [31,35].

In summary, we have described the cloning and characterization of a permease of *P. chrysogenum*, specific for aromatic amino acids and leucine. *S. cerevisiae* proved to be a suitable host for overexpression and characterization.

Acknowledgements

S. cerevisiae strain M4054 was kindly provided by M.C. Kielland-Brandt (Department of Physiology, Carlsberg Laboratory, Copenhagen, Denmark). We thank Dr. M. van der Kamp and Dr. J. Kiel for valuable discussions. This work was support by the European Community (grant no QLK3-CT-1999-00729).

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