Amino acid transport in Penicillium chrysogenum in relation to precursor supply for beta-lactam production

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Chapter 5

Random mutagenesis of PcDIP5 and selection for improved α-aminoacidipate transport

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Abstract

Saccharomyces cerevisiae is unable to utilize α-aminoacidipate as a primary nitrogen source unless LYS2 or LYS5 is disrupted. In S. cerevisiae strain M4276, deficient in uptake of acidic amino acids, the LYS2 gene was deleted, resulting in strain M4600. The acidic amino acid permease gene of Penicillium chrysogenum, PcDIP5, was expressed in M4600 to allow uptake and utilization of α-aminoacidipate as primary nitrogen source. In an attempt to improve the uptake capacity of PcDip5 for α-aminoacidipate, a random mutagenesis was performed by error-prone PCR. Cells of strain M4600 were transformed with the mutagenized PcDIP5 and were selected for faster growth on plates with a low concentration of α-aminoacidipate. Mutant strains were selected with faster growth characteristics but these did not relate to an improved α-aminoacidipate transport activity.
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Introduction

Penicillin biosynthesis in *Penicillium chrysogenum*, which starts with the condensation of three amino acid precursors L-α-aminoacidipate, L-cysteine and L-valine, to form the tripeptide ACV (1), is stimulated by external addition of α-aminoacidipate (59, 81). The uptake of α-aminoacidipate is mediated by active transport into the cells via the acidic amino acid permease PcDip5 and the general amino acid permease PcGap1 (208). PcDip5 is a high affinity transporter of L-glutamate and L-aspartate \( (K_m = \text{approx. } 35 \ \mu M) \), whereas α-aminoacidipate is transported with low affinity \( (K_m = \text{approx. } 800 \ \mu M) \). PcGap1 can transport all amino acids and has a higher affinity for α-aminoacidipate \( (K_m = 230 \ \mu M) \).

In this work, we attempt to improve PcDip5 for α-aminoacidipate transport by a random mutagenesis and a positive selection strategy. An improvement of the transport affinity might be beneficial to the penicillin production capacity as it would allow more efficient savaging of extracellular α-aminoacidipate, i.e., a better retention in the cell. Little is known about structure-function relationships in members of the AAP family, to which PcDip5 belongs (6, 208). A site-directed mutagenesis study on the major proline permease gene *prnB* of *Aspergillus nidulans* has implicated two residues that are present in TMS 6 to be important for substrate selectivity or binding (201). In a random mutagenesis strategy on *CAN1*, the arginine permease gene of *S. cerevisiae*, mutants were selected that had acquired the ability to mediate citrulline uptake. These mutations were located in transmembrane segments 3 and 10, as well as in cytoplasmic and extracellular loops. Most of the mutations are at conserved positions in the AAPs family, but there is no evidence that they are directly involved in substrate recognition, i.e., they may affect the transport activity and specificity in an indirect way (160). Considering the large difference in affinity of PcDip5 for α-aminoacidipate and glutamate but at the same time the similarity in structure of these substrates, we envision the possibility to alter PcDip5 in such a way that its affinity or capacity for α-aminoacidipate transport is increased. For positive selection of improved transport, an assay may be employed in which growth is dependent on α-aminoacidipate uptake via *PcDIP5*. In earlier work, PcDip5 was characterized by the functional expression of *PcDIP5* in *S. cerevisiae* M4276, which lacks the genes encoding the acidic amino acid permease *DIP5* and the general amino acid permease.


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GAP1 (159, 208). To select for improved α-aminoadipate transport, a library of randomly mutagenized PcDIP5 can be expressed in S. cerevisiae M4276 and cells may be selected for faster growth on plates with a limiting amount of α-aminoadipate as sole nitrogen source. However, to enable S. cerevisiae to use α-aminoadipate as a nitrogen source, either LYS2 or LYS5, together encoding α-aminoadipate reductase, has to be disrupted (28, 233). α-Aminoadipate is an intermediate of the lysine biosynthesis pathway and is converted into α-aminoadipate semialdehyde by the activity of α-aminoadipate reductase. Normally, external addition of α-aminoadipate is toxic for S. cerevisiae, probably due to an increase of the intracellular α-aminoadipate semialdehyde concentration which might be toxic to the cells. If LYS2 or LYS5 is deleted and α-aminoadipate is added to the medium, α-aminoadipate semialdehyde is no longer formed, α-aminoadipate will accumulate and will be shuttled into a transaminase pathway. Probably, the amino group of α-aminoadipate is transferred to α-ketoglutarate to form α-ketoacidipate and glutamate, of which the latter will go into nitrogen assimilation. Hereby, α-aminoadipate serves as primary nitrogen source, while cells are now lysine auxotroph and have to be provided with lysine for protein synthesis (233). Here, we describe the construction of a new, LYS2 negative and acidic amino acid uptake deficient strain of S. cerevisiae and the use of it for positive selection of PcDip5 mutants improved in α-aminoadipate transport.

Material and methods

Strains and media. The strains used in this study are S. cerevisiae M4276 (MATα, ura3, Δgap1, Δdip5) (159) and M4600 (MATα, ura3, Δgap1, Δdip5, Δlys2), which was directly derived from strain M4276 by the deletion of the LYS2 gene. Cells were grown in minimal medium containing 0.1 % L-proline (MP) (159), glutamate (0.5 g/L) or α-aminoadipate at indicated concentrations as nitrogen source and supplemented with uracil (20 mg/L) when needed. For growth of strain M4600, 0.3 g/L lysine was added to complement for lysine auxotrophy. YPG (1 % yeast extract, 2 % peptone and 2 % glucose) was used for non-selective growth. E. coli DH5α was used for all cloning steps, carried out as described in (174).
Deletion of LYS2 in S. cerevisiae M4276. A LYS2 deletion cassette was constructed by using 2 oligonucleotides, one containing 60 bp of the 5’ end of LYS2 and 20 bp of the 5’ end of the zeocin resistance cassette/gene: ATGACTAACGAAAAGGTCTGGATAGAGAAGTTGGATAATCCAACTCTTTT
 AGTGTTACCAGGTCTAGCTTGTTCGATCC, and one containing 60 bp of the 3’ end of LYS2 and 20 bp of the 3’ end of the zeocin resistance cassette: TTAAGCTCCGAGCTTTCCACGACGACCCACCTGAAGCAACTAGACCTTA
 TTTGCCGCTGCTACATGTTGGTCTCCAGC. A PCR was performed using the plasmid pREMI-Z (217) containing the zeocin resistance cassette, as template DNA. The 4.3 kb product was gel-purified and transformed to S. cerevisiae M4276 cells, according to Gietz et al. (62). Cells were selected for zeocin resistance on YPG plates containing 100 mg/L zeocin. Colonies appeared after two days and were transferred to liquid YPG medium for DNA isolation. Deletion of LYS2 was tested by PCR using oligonucleotides annealing to the 5’ flanking region of LYS2 (forward primer) and the zeocin resistance gene (backward primer), as well as oligonucleotides annealing to LYS2, amplifying an internal LYS2 fragment.

The M4276 lys2 strain, denoted M4600, was transformed with yEX-PcDIP5 (208) to enable uptake of α-aminoadipate, and with yEX-C, the empty vector, as a control. Transformants were tested on minimal medium plates containing 1 g/L L/D-α-aminoadipate, 600 mg/L L-lysine and 0.2 mM CuSO4 for induction of expression of PcDIP5 (208). Cells containing yEX-PcDIP5 were able to grow, whereas the control cells were not. To show that lysine in the medium was not used as a primary nitrogen source instead of α-aminoadipate, M4600 cells were streaked on plates containing 600 mg/L L-lysine as sole nitrogen source.

Random mutagenesis of PcDIP5 by error prone PCR. Random mutagenesis was carried out basically as described by Leemhuis et al. (109). The PcDIP5 gene (plus additional 200 bp plasmid DNA up- and downstream PcDIP5) was amplified from yEX-PcDIP5 (208) using the following primers: Dipmut F, GGTACCGGTCCGAATTCC and Dipmut R, GGCCTGTTTACTCACAGGC. The PCR mixture contained the following: 10 mM of dATP, dCTP, dGTP and dTTP, 1 X Taq DNA polymerase buffer (Roche), 2 mM MgCl2, 0.2 mM MnCl2, 0.2 μM of each primer, 2 units Taq polymerase (Roche) and 50 ng template DNA in a total volume of
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50 µL. The PCR reaction was performed for 30 cycles: 30 sec at 94°C, 40 sec at 55°C and 2 min at 72°C. The PCR product was analyzed by gel-electroforesis and purified and digested with XhoI, located 27 bp downstream the start codon, and NotI, located 136 bp downstream the stop codon of PcDIP5. The resulting 1.8 kb fragment was ligated in the linearized yEX-C vector resulting from XhoI and NotI digested yEX-PcDIP5. For optimal ligation conditions, 5 % PEG-8000 (final conc.) was used in the ligation mixture. Electrocompetent XL1-Blue cells (Stratagene) were transformed with purified DNA from the ligation mixture, yielding approx. 120,000 transformants. Plasmid DNA was isolated for transformation of S. cerevisiae M4600.

Transformation of M4600 and selection for improved growth at limiting α-aminoadipate concentration. S. cerevisiae M4600 was transformed according to Gietz et al (62), resulting in at least 100,000 transformants. Cells were spread on minimal medium plates containing 0.1 g/L L/D-α-aminoadipate, 0.3 g/L L-lysine and 0.2 mM CuSO₄ for induction of PcDIP5 expression. Plates were incubated for 10 days at 30°C. Colonies of apparent larger size were transferred to fresh plates with the same medium and plates were incubated for 10 days at 30°C. Plasmid DNA was isolated from these clones by standard methods, transformed to E. coli DH5α for amplification and finally reintroduced into strain M4600 and also M4276. M4600 transformants were plated on minimal medium as described above, i.e. with 0.1 g/L L/D-α-aminoadipate as primary nitrogen source. M4276 transformants were used in a transport assay to directly test α-aminoadipate uptake.

Transport assays. Strain M4276 was transformed with plasmid DNA obtained from 8 M4600 transformants showing increased growth on plates with a limiting concentration of α-aminoadipate as primary nitrogen source. Transformants were grown overnight in minimal proline (MP) medium, then diluted 10 times in fresh MP medium, supplemented with 0.2 mM CuSO₄ for induction of the copper inducible promoter pCUP1, upstream PcDIP5. After 5 hours of incubation, cells were harvested by centrifugation, washed once with minimal medium without a nitrogen source and resuspended in the same medium. Transport assays using ³H-labelled L/D-α-aminoadipate were carried out as described by Trip et al., 2002 (209) using concentrations of 25 µM up to 1 mM.
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Results

Deletion of LYS2 in *S. cerevisiae* M4276. In earlier work, transport of α-aminoadipate, glutamate and aspartate by PcDip5 was studied in *S. cerevisiae* strain M4276 (Δgap1, Δdip5) by functional expression of the *Penicillium chrysogenum DIP5 (PcDIP5)* and transport assays using radioactively labeled amino acids (159, 208). Since these assays are performed on a minute scale, the growth inhibiting or even toxic effect of externally added α-aminoadipate to *S. cerevisiae* cells (28) does not interfere with the results. Here we aimed to screen a library of PcDip5 mutants for increased α-aminoadipate transport. At limiting α-aminoadipate concentration, growth of *S. cerevisiae* is dependent on the α-aminoadipate uptake capacity via PcDip5. Under conditions that transport is limiting, improved transport characteristics may lead to faster growth. In order to allow growth of *S. cerevisiae* on α-aminoadipate as primary nitrogen source, the LYS2 gene needs to be disrupted in strain M4276. Since LYS2 encodes the α-aminoadipate reductase, an enzyme of the lysine biosynthesis pathway, cells lacking this enzyme are lysine auxotroph and need to be provided with lysine (233). *S. cerevisiae* M4276 was transformed with a LYS2 deletion cassette, containing the zeocin resistance gene as a selectable marker and two 60 bp flanking regions of LYS2. Zeocin resistant colonies were tested for the LYS2 deletion by PCR using appropriate primers and appeared all to be LYS2*. In addition, cells were tested phenotypically by plating on minimal medium plates, supplemented with uracil, with and without lysine. The zeocin resistant cells did not grow without lysine, whereas control cells (M4276) grew normally (Fig. 2A). The new strain, denoted M4600, was tested for its ability to use α-aminoadipate as a primary nitrogen source. Cells were transformed with yEX-PcDIP5 and with yEX-C (empty vector) as a negative control. Transformants were plated on minimal medium containing 1 g/L L/D-α-aminoadipate, 0.3 g/L L-lysine and 0.2 mM CuSO4 for induction of PcDIP5 expression. Cells containing yEX-PcDip5 grew normally, whereas cells containing the empty vector did not grow at all (Fig. 1). To test whether the lysine in the medium was not used as the primary nitrogen source, cells of M4600 were plated on minimal medium containing 0.6 g/L L-lysine as a sole nitrogen source. Very poor growth was observed, even after 5 days, indicating that lysine is not a good nitrogen source for *S. cerevisiae*. This result can also be taken as evidence that the presence of lysine does
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**Figure 1.** Growth of *Saccharomyces cerevisiae* strain M4600 (M4276 with deleted LYS2 gene) with α-aminoacidipate as primary nitrogen source is rescued by the heterologous expression of PcDIP5. Plates contain minimal medium with 1 g/L D/L α-aminoacidipate, 0.6 g/L L-lysine to complement for lysine auxotrophy of strain M4600 and 0.2 mM CuSO4 for PcDIP5 expression. In M4276 yEX-PcDIP5 (upper left part), α-aminoacidipate is taken up via PcDip5, but will be toxic to the cells. With the LYS2 deletion, M4600 yEX-PcDIP5 can take up and assimilate α-aminoacidipate and grow (upper right part). Without expression of PcDIP5 (M4600 yEX-C, right, below), cells cannot take up α-aminoacidipate and cannot grow on α-aminoacidipate as primary nitrogen source.

not significantly interfere with α-aminoacidipate as a primary nitrogen source (Fig. 2B). Using low concentrations of lysine (30 mg/L which should be sufficient to satisfy the lysine auxotrophy according to Zaret et al., 1985 (233)) resulted in strongly reduced growth (Fig. 2A). This might be explained by a reduced uptake of lysine due to the absence of the general amino acid permease in strain M4600, although the basic amino acid transport systems (Can1, Lyp1 and Alp1) which are still present in this strain may substitute for the Gap1 deficiency.

**Random mutagenesis of PcDIP5 by error prone PCR.** In order to generate a mutant of PcDip5 with improved α-aminoacidipate transport capacity or affinity, random mutagenesis was applied using error prone PCR. A mutant PcDip5 library was created using 0.2 mM MnCl2 in an otherwise standard PCR. Ligation of the PCR
Figure 2. Lysine auxotrophy in *S. cerevisiae* M4600 (M4276 with deleted LYS2 gene). (A) *S. cerevisiae* M4600 needs a relatively high concentration of externally added lysine to fully complement for lysine auxotrophy. Plates contain 5 g/L NH$_4$SO$_4$ as nitrogen source and 30 and 600 mg/L L-lysine. (B) Lysine is a poor nitrogen source for *S. cerevisiae* M4600. After 5 days of incubation, hardly any growth was observed with up to 0.6 g/L of L-lysine as a sole nitrogen source.

Product in the expression vector yEX-C and transformation to *E. coli* resulted in approx. 120,000 clones. Plasmid DNA was isolated and transformed to *S. cerevisiae* M4600, yielding approximately 100,000 transformants. To test the effect of the mutagenesis on the activity of PcDip5, transformants were tested for their ability to grow on plates with minimal medium and 0.5 g/L L-glutamate as sole nitrogen source. Of 50 tested clones, 12 did not grow at all or very slowly on this medium, indicating that about 75 % of the mutant PcDip5 clones retained normal glutamate transport activity.
Selection for improved α-aminoadipate uptake. S. cerevisiae M4600 was transformed with the mutant library of PcDip5, resulting in about 100,000 transformants. Cells were plated on minimal medium containing 0.05 g/L L/D-α-aminoadipate, 0.3 g/L L-lysine and 0.2 mM CuSO₄, and plates were incubated for 10 days. A number of colonies with larger size than the majority of colonies appeared and these were transferred to fresh plates for isolation of individual clones. From these clones, plasmid DNA was isolated and transformed back to strain M4600 as well as M4276. Strain M4600 transformed with the isolated plasmid DNA, however, did not show an enhanced growth in comparison with M4600 expressing wild-type PcDIP5. Likewise, M4276 cells transformed with the same plasmid did not show increased α-aminoadipate uptake when tested at 25 μM and 1 mM concentration.

Discussion

The acidic amino acid permease PcDip5 shows a high affinity for aspartate and glutamate (Kₘ, 35 μM), but a low affinity for α-aminoadipate (Kₘ, 800 μM), although the latter only differs from glutamate in having a side chain one that is one CH₂-group longer. This chapter describes an attempt to randomly mutagenize PcDip5 and select for improved α-aminoadipate transport using a plate assay in which growth of S. cerevisiae is dependent on α-aminoadipate transport via PcDip5. For this purpose, a new strain of S. cerevisiae was constructed, i.e., M4600. This strain is derived from strain M4276, deficient in uptake of acidic amino acids, by deleting LYS2, encoding aminoadipate reductase. This deletion blocks lysine biosynthesis, but enables the cells to use α-aminoadipate as a primary nitrogen source. In this strain, a library of randomly mutagenized PcDIP5 was expressed and cells were grown on plates with a limiting concentration of α-aminoadipate as primary nitrogen source. However, PcDip5 clones isolated from colonies with a clearly larger size than the vast majority of colonies, did not show increased α-aminoadipate uptake after further analysis. The larger size of colonies may have been due to non-specific, possibly spontaneous mutations that provide the cells a growth advantage on the specific minimal medium. In general, selection on basis of colony size as an indicator for rate of transport of a growth limiting substrate might be too indirect.
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In this work we tried to improve PcDip5 for α-aminoadipate transport by random mutagenesis with two intentions, firstly to improve α-aminoadipate (re)uptake in P. chrysogenum and secondly to gain inside in structure/function relationships in AAPs. For this purpose, a random mutagenesis approach seems to be preferred provided that a suitable screening method can be applied in which the desired change in transport characteristic leads to a much more distinct phenotype than a difference in colony size. A more rational approach is site-directed mutagenesis, but currently it is unclear what residues should be targeted for such an approach. A first step could be the construction of chimaeric genes, in which parts of genes of AAPs with different substrate specificity are combined. This may lead to a better insight in what regions/domains of the transporter define substrate specificity.

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