Uracil and beta-alanine degradation in
*Saccharomyces kluyveri*
- Discovery of a novel catabolic pathway

Ph.D. Thesis

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PREFACE

The thesis presented here is a part of the requirements for the Ph.D. degree under the "Chemistry and Biotechnology Programme" at the Technical University of Denmark (DTU). Since October 2002, I have been enrolled at BioCentrum-DTU. The study was financed by DTU and has been carried out under supervision of former Associate Professor at BioCentrum-DTU, (now Professor at Department of Cell and Organism Biology, Lund University, Sweden) Jure Piskur.

I would like to thank my supervisor, Jure Piskur, for his support and guidance throughout the project. His critical questions kept me motivated and focused. I would also like to thank both permanent and temporary people from "Jure’s Lab" (before he decided to cross Oresund). Thanks to Anders, Birgit, Dorte, Ela, Gloria, Kostya, Li, Michael, Olena, Silvia, Yuriy and to the former members of the lab, Jesper, Lise, Rikke, Trine, whom I met briefly in the lab, but numerous times afterwards at Jure’s social events. Special thanks to the two super-secretaries Anita and Hanne at building 301. From the newer Swedish version of "Jure’s Lab", I would like to thank Olof, Anna, Patrick, Antonios, Marita Cohn and her group (Jenny and Eimantas) and the rest of "Biologihuset". Special thanks to Klaus D. Schnackerz for the many trips to the lab (both sides of the sound). Thanks to Anders Hofer for having me in his lab, and to Reza and Munender in Anders’ lab for the many laughs. And thanks to Vladimir Domkin for his enthusiasm and interesting discussions on alternative reactions and compounds.

Some experimental parts of this thesis have been performed by other people and this is specifically acknowledged in each chapter.

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Copenhagen, May 2006

Gorm Andersen
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<th>Common name</th>
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<td>5-FC</td>
<td>5-fluorocytosine</td>
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<td>5-fluoro-2'deoxyuridine 5'-monophosphate</td>
<td></td>
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<td>5-FdUTP</td>
<td>5-fluoro-2'deoxyuridine 5'-triphosphate</td>
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<td>5-fluouracil</td>
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<td>5-fluouridine</td>
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<tr>
<td>5-FUTP</td>
<td>5-fluouridine 5'-triphosphate</td>
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<td>alpha-ketoglutarate, 2-oxoglutarate</td>
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<td>beta-alanine</td>
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<td>beta-ureidopropionate</td>
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<td>methylmalonic semialdehyde</td>
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<td>pyridoxal-5'-phosphate</td>
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<td>PRPP</td>
<td>5'-phosphoribosyl-1'-pyrophosphate</td>
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<td>uridine diphosphate</td>
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<td>UMP</td>
<td>uridine monophosphate</td>
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<td>UTP</td>
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<td>Abbreviation</td>
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<td>carbamoyl-phosphate:L-aspartate carbamoyltransferase</td>
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<td>4-aminobutanoate:2-oxoglutarate aminotransferase</td>
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<td>UMP:phosphate phospho-alpha-D-ribosyltransferase</td>
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<td>uridine ribohydrolase</td>
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<td>URK</td>
<td>ATP:uridine 5'-phosphotransferase</td>
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ABSTRACT

It is generally believed that pyrimidine degradation is initiated by either a reductive or an oxidative step! Are there really no alternatives? Degradation of pyrimidines is of a great importance in humans. Defects in the genes involved in the corresponding pathway cause severe symptoms, and especially in cancer patients a combination of gene defects and chemotherapy with e.g. 5-fluorouracil (a widely used anti-cancer drug) can have fatal consequences. The first step in the catabolic pathway performed by dihydropyrimidine dehydrogenase (DHPDH), has been found in mammals, insects, plants and bacteria. This first step has so far not been found in the fungi kingdom, but two genes (PYD2 and PYD3) encoding the two subsequent steps in the catabolic pathway, have previously been characterized in the yeast, Saccharomyces kluveyri. In this thesis, the origin of the uracil degradation pathway in yeast, and the genetic background for uracil and beta-alanine (BAL) catabolism in S. kluveyri, were investigated.

The ability to use uracil, dihydrouracil (DHU), beta-ureidopropionate (BUP) and BAL as nitrogen source was studied in thirty-eight yeast species selected to cover the "Saccharomyces complex", which originated more than 200 mill. years ago. Uracil, DHU and BUP degradation were found to be linked in almost all the thirty-eight yeast species tested, and the pathway was apparently lost after the whole genome duplication. The ability to use BAL as sole nitrogen source was not linked to the three others, and was lost more or less randomly. In order to study the genetic background of uracil degradation in S. kluveyri a number of mutants defective in uracil degradation were isolated, and the defects were found to belong to six loci (PYD11,12,13,14,15,16). None of these loci were allelic to the previously described PYD2 and PYD3 loci, and all of the mutants could use DHU and BUP as sole nitrogen source. Targeted disruption of all eight loci showed that uracil is degraded by a pathway consisting of the PYD1X genes, while DHU is degraded by a pathway consisting of PYD2 and PYD3. Surprisingly, uracil is degraded via UMP, and urea is an intermediate. A new gene, termed PYD4, found to be involved in DHU degradation was isolated. PYD4 encodes a pyridoxal-5'-phosphate-dependent aminotransferase, which shows similar activity and substrate specificity as mammalian BAL/gamma-aminobutyrate aminotransferase (BAL/GABA-AT) [EC 2.6.1.19]. S. kluveyri also has an UGA1 encoded GABA-AT [EC 2.6.1.19], which is specific for GABA. The original gene was apparently duplicated, thus giving PYD4 and UGA1, less than 200 mill. years ago.
DANSK RESUME

Det første skridt i nedbrydningen af pyrimidiner er enten en reduktiv eller en oxidativ reaktion! Er der virkelig ingen alternativer? Nedbrydningen af pyrimidiner har stor betydning i mennesket. Genetiske defekter i den tilhørende pathway medfører alvorlige symptomer og specielt i for kræftpatienter, som får pyrimidinbaseret kemoterapi (f.eks. 5-fluorouracil, som er et meget anvendt kræfthæmmende stof), kan disse defekter få letale konsekvenser. The første trin i den katabolske pathway udført af dihydropyrimidine dehydrogenase (DHPDH), er blevet fundet i pattedyr, insekter, planter og bakterier. Dette første skridt er dog endnu ikke blevet fundet i svampe riget, men to gener (PYD2 og PYD3), som koder for de efterfølgende trin i den katabolske pathway, er tidligere blevet karakteriseret i gøren, Saccharomyces kluyveri. I denne afhandling, bliver oprindelsen af uracil nedbrydningspathway’en i gør og de genetiske forudsætninger for uracil og beta-alanine (BAL) katabolisme i S. kluyveri undersøgt.

Evnen til at bruge uracil, dihydrouracil (DHU), beta-ureidopropionate (BUP) og BAL som nitrogenkilde blev studeret i 38 gør arter. Disse var udvalgt, så de dækkede “Saccharomyces komplekset”, som opstod for mere end 200 mill. år siden. Evnen til at nedbryde uracil, DHU og BUP var sammenkædet i næsten alle 38 gør arter, som blev testet, og pathway’en blev tilsyneladende tabt efter genom duplikationen. Evnen til at bruge BAL som eneste nitrogenkilde var uafhængig af de tre andre, og blev tabt mere eller mindre tilfældigt. For at studere de genetiske forudsætninger for nedbrydning af uracil i S. kluyveri, blev et antal mutanter, som var defekte i nedbrydningen af uracil, isoleret, og defekterne tilhørte seks loci (PYD11,12,13,14,15,16). Ingen af disse var identiske med de to tidligere beskrevne, PYD2 og PYD3 loci og alle mutanterne kunne bruge DHU samt BUP som eneste nitrogenkilde. Målrettet ødelæggelse af alle otte loci viste, at uracil bliver nedbrudt via en pathway bestående af PYD1X generne, mens DHU bliver nedbrudt via en pathway bestående af PYD2 og PYD3. Overraskende nok, så blev uracil nedbrudt via UMP og urea var et intermediat. Et nyt gene, kaldet PYD4, som er involveret i nedbrydningen af DHU, blev isoleret. PYD4 koder for en pyridoxal-5’-fosfat-afhængig aminotransferase, som har sammenligneligt aktivitet og substratspecificitet som pattedyr BAL/gamma-aminobutyrate aminotransferase (BAL/GABA-AT) [EC 2.6.1.19]. S. kluyveri har også en UGA1 kodet GABA-AT [EC 2.6.1.19], som er specifik for GABA. Det oprindelige gen blev tilsyneladende duplikeret for mindre end 200 ,ill. år siden, hvilket resulterede i PYD4 og UGA1.
GENERAL INTRODUCTION

Pyrimidines are the key components or precursors in many biomolecules. The metabolism of pyrimidines (and purines) and their intercellular pool sizes greatly influence a number of normal cellular metabolic pathways, and are therefore central for proper functioning of the cell (Reviewed in Bianchi, 1998).

Pyrimidine metabolism is split into three parts: anabolic, salvage and catabolic (Reichard, 1988). Figure 1 is an illustration of the pyrimidine metabolism around the central metabolite, uridine-5’-monophosphate (UMP).

![Pyrimidine metabolism diagram](image)

Figure 1: Pyrimidine metabolism. The three major pathways involved in pyrimidine metabolism, biosynthesis, salvage and degradation are indicated. UMP synthesized or salvaged gets further metabolized and incorporated into DNA, RNA and phospholipids, and is directly involved in polysaccharide formation. The intermediate, beta-alanine, of pyrimidine degradation, is a constituent of pantothenate.

The anabolic pathway, where UMP is synthesized from simple organic compounds via six enzymatic steps involving carbamyl phosphatesynthase II (CPSII), aspartate transcarbamylase (ATC), dihydroorotase (DHOT), dihydroorotate dehydrogenase
General introduction

(DHODH), orotate phosphoribosyltransferase (OPRT) and orotidine 5’-phosphate decarboxylase (OMPDC) is ubiquitous in the biosphere, while the salvage pathway, where premade nucleobases and nucleosides are transported (via specific transporters), phosphoribosylated by uracil phosphoribosyltransferase (UPRT) or phosphorylated by uridine kinase (URK) to the UMP level, is found in higher eukaryotes, plants and several microorganisms. While the genes of the anabolic and salvage pathways seems to be conserved, the ability to catabolize pyrimidines has evolved in two alternative routes; (I) the reductive pathway employing the three consequetive activities of dihydropyrimidine dehydrogenase (DHPDH), dihydropyrimidinase (DHP) and beta-ureidopropionase (UP), or (II) the oxidative pathway employing the three consequetive activities of uracil dehydrogenase (UDH), barbiturase (BAA) and beta-ureidomalonase (UM). These catabolic pathways, which both use uracil or thymine as first compound, has been found in much fewer organisms than the de-novo and salvage pathways. The reductive is by far the most abundant of the two, and it has been fully or partly described in mammals, insects, plants, yeast and bacteria. With the expanding repertoire of genome sequences available the number of organism possessing this pathway is "growing". The oxidative pathway has only been found in a few bacteria, and only two genes belonging to this pathway have been cloned. These genes have no homologs in any other sequenced organism, but share some identity to enzymes involved in other metabolic pathways. Beside the role as a nitrogen source for microorganisms, the end-product of uracil degradation, beta-alanine (BAL), is a crucial component in the biosynthesis of pantothenate in many organisms (excluding mammals). Several other pathways are found in nature which provide BAL. Further degradation leads to malonic semialdehyde (MSA) and methylmalonic semialdehyde (MMSA), which in turn gets coupled to CoA.

The reductive catabolic pathway has recieved a lot of attention since it has been found to be of clinical interest. It’s a key determinant in the cytotoxicity of some pyrimidine based anti-cancer drugs, and inborn errors in the enzymes of the catabolic pathway has been identified from a number of patients leading to primarily neurological defects and a lot of research is focusing on optimizing the drug administration by determining the patients susceptibility to the drug pretreatment.
Chapter 1

Yeast has for a long time been used as a model organism to study basic cellular processes. Fundamental things like telomeres and aging, DNA replication/repair mechanisms and function of peroxisomes have been studied intensely in yeast, and brought much insight for the rest of the eukaryotic world (Aylon and Kupiec, 2004; Goldman and Kafer, 2004; Teixeira and Gilson, 2005; van Roermund et al., 2003). With the growing number of yeast genome sequences comparative genomics and evolution of not just genes or pathways but chromosome and genome dynamics can now be studied by global approaches. Information about intergenic sequences can be explored. There are still new areas, where yeast are becoming an important source of new information. Like fx. pyrimidine catabolism as mentioned before, where the yeast \( S. \ kluyveri \) is being developed as a model organism to study this pathway.

For many years it has been attempted to describe the catabolism of pyrimidines in the fungi kingdom. It was evident that members of the group could degrade pyrimidines (by using them as sole nitrogen sources), but the way it was degraded did not always match with either of the two known catabolic pathways, so some rather exotic pathways were postulated. It was first in 1998, when the non-conventional yeast, \( Saccharomyces \ kluyveri \), was used in a systematic study, that a clear evidence of the reductive pathway was found (Gojkovic et al., 1998).

AIMS AND OUTLINE

The idea of this Ph.D. project was to elucidate the pyrimidine degradation in yeast. The primary goal was identification of the first enzymatic step in the pathway, since the two subsequent steps had already been relatively well characterized (Gojkovic et al., 2000; Gojkovic et al., 2001). As it will be evident from Chapter 4, things have been taking a turn and some new and exciting discoveries have been made. Also questions like BAL degrading enzyme(s), the diversity of the pathway within the yeast species and the evolutionary relationship between the anabolic and catabolic pathways were in focus.

In the following chapter (Chapter 2) the present status on pyrimidine catabolism and end-product metabolism is summarized. The \textit{de-novo} pathway is interesting in this context, because the three enzymes in this pathway, namely ATC, DHO and DHODH share a great deal of identity with the three enzymes of the reductive pathway, UP,
DHP and DHPDH, respectively, and it seems the two pathways share the same origin. Since the organism studied in this work is a yeast, a section on yeast phylogeny, genomics and nitrogen metabolism is presented, and another part is dedicated specifically to *S. kluyveri* and the historical background of pyrimidine degradation in yeast/fungi. The following three chapters are the results obtained from this Ph.D.-project. Chapter 3 describes the degradation of uracil and intermediates of the reductive pathway by thirty-eight yeast strains, representing the whole *Saccharomyces* complex including the genera *Saccharomyces*, *Arxiozyma*, *Kluyveromyces*, *Candida*, *Zygosaccharomyces*, *Torulaspora* and *Hanseniaspora*. The work has been accepted for publication: **Andersen, G., Merico, A., Björnberg, O., Andersen, B., Schnackerz, K.D., Dobritzsch, D., Piškur, J. & Compagno, C.** (2006) Catabolism of pyrimidines in yeast: a tool to understand degradation of anti-cancer drugs. *Nucleosides Nucleotides Nucleic Acids*. Chapter 4 is a presentation of a novel uracil degrading pathway found in yeast and bacteria. It is based on mutagenesis and gene knockout studies of the uracil pathway, and the results from the work done at Department of Medical Biochemistry and Biophysics, Umeå University in A. Hofer’s lab. In Chapter 5, the identification and cloning of the *SkPYD4* and *SkUGA1* genes, which encodes a BAL and gamma-aminobutyrate (GABA) aminotransferase (BAL-AT, GABA-AT) respectively. The gene products were overexpressed, purified and characterized with newly developed coupled enzymatic assay. Also Uga1p (GABA-AT) from *S. cerevisiae* and *Schizosaccharomyces pombe* were overexpressed, purified and partly characterized. This chapter will be submitted as a manuscript in May, 2006. Finally, in Chapter 6 is a general discussion with a summary of the results obtained and some concluding remarks on pyrimidine degradation in yeast.

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DE NOVO UMP BIOSYNTHESIS

The de novo biosynthesis of uridine monophosphate is composed of 6 enzymatic activities (Figure 1). Actually the first step, by carbamoyl phosphate synthase II (CPSII, EC 6.3.5.5), is composed of two catalytic activities, a glutamine-dependent amidotransferase and a synthetase, but is usually considered as one enzymatic unit (Simmer et al., 1990). CPSII catalyze the formation of carbamoyl phosphate from glutamine, ATP, CO$_2$ and H$_2$O. Carbamoyl phosphate and aspartate are by aspartate transcarbamylase (ATC, EC 2.1.3.2) turned into ureidosuccinate, which then gets dehydrated and circularized by dihydroorotase (DHOT, EC 3.5.2.3) to form dihydroorotate (DHO). In higher eukaryotes, this part of the pathway has evolved in a way that individual enzymatic steps have become physically linked in a multi-functional enzyme (Figure 2). In prokaryotes these enzymatic steps are encoded by four different genes, (pyrAa, pyrAb, pyrB, pyrC). In Dictyostelium discoideum, Drosophila melanogaster and mammals, the multi-functional enzyme, (called PYR1-3, rudimentary and CAD, respectively), performs the first three enzymatic activities, CPSII, ATC and DHOT. In yeast this enzyme is called Ura2p, but even though it has

![Figure 1: Pyrimidine de-novo biosynthesis. The figure is showing the enzymatic reactions involved in UMP biosynthesis. Abbreviations used are, CPSII: Carbamoyl phosphate synthase II, ATC: Aspartate transcarbamoylase, DHOT: Dihydroorotase, DHODH: Dihydroorotate dehydrogenase, OPRT: Orotate phosphoribosyltransferase, OMPDC: Oridine-5’-monophosphate decarboxylase.](image-url)
high identity to CAD, it contains an inactive DHOT, and instead has a separate enzyme performing the DHOT activity (Ura4p) (Denis-Dupil, 1989; Souciet et al., 1989).

The enzyme containing the inactive DHOT is believed to be ubiquitous in the fungal kingdom (Aleksenko et al., 1999). The fourth step, oxidation of DHO to orotate, is performed by dihydroorotate dehydrogenase (DHODH, EC 1.3.3.1). This is in eukaryotes done either within the mitochondrial matrix or in the cytoplasm, and the localization is dependent on two different types of DHODH (mtDHODH and cytDHODH). Apparently the cytoplasmic type is involved in the ability of a microorganism to grow under anaerobic conditions, where the respiratory chain is uncoupled (Gojkovic et al., 2004). Also some bacteria have two different types of DHODH enzymes (reviewed in Kilstrup et al., 2005). The orotate is phosphoribosylated by orotate phosphoribosyltransferase (OPRT, EC 2.4.2.10) to orotidine monophosphate (OMP), which in turn is decarboxylated to UMP by OMP-decarboxylase (OMPDC, EC 4.1.1.23). These last two steps are again organized in the same protein in D. melanogaster and mammals (UMP synthase), while yeast have them separated (Ura5p, Ura10p and Ura3p respectively) (Figure 2). Ura5p and Ura10p are isoenzymes and both are functional (de Montigny et al., 1990).

**SALVAGE OF PYRIMIDINES**

The pyrimidine *de novo* pathway is found in almost all known organisms, and it is efficient enough to supply the needed pyrimidines (UMP) for growth, although a lot of organisms have a system for salvaging premade pyrimidines from the
surroundings, and thereby saving time, energy and building materials. This salvage pathway is composed of elements that sense and transport extracellular pyrimidines into the cytosol, where they quickly get transformed to the riboside monophosphate level (Figure 3). Some of the first identifications of genes involved in yeast pyrimidine salvage was done on the basis of 5-fluoropyrimidine resistance (Jund and Lacroute, 1970).

Figure 3: Pyrimidine salvage pathway in *S. cerevisiae*. The figure shows the transport and conversion of different pyrimidines into UMP. Fur4p: uracil permease, Fui1p: uridine permease, Fcy2p: cytosine/cytidine permease, Fcy1p: cytosine deaminase, Cdd1p: cytidine deaminase, Urh1p: uridine hydrolase, Urk1p: uridine kinase, Fur1p: uracil phosphoribosyl transferase. (Kurtz et al., 1999).

The cytotoxic activity of the analog is exerted by the salvage pathway. This selectivity offered by the 5-fluoropyrimidines has been used to identify all enzymes involved in the pyrimidine-base salvage in yeast. Seven alleles were found to be involved in resistance to one or more of the three tested analogs, 5-fluorouracil (5-FU), 5-fluorocytosine (5-FC) and 5-fluorouridine (5-FUri). Now eight genes involved in pyrimidine salvage pathway in yeast are known (Table 1), and it has been shown that the pathway primarily leads to UMP formation (Kurtz et al., 1999). The only exception is in the case of cytidine, where a limited amount is converted to CMP via Urk1p, while the majority is converted to UMP. Cytidine transport is dependent on the *FCY2* encoded purine/cytosine transporter.
Transporters
There are three pyrimidine transporter encoding genes in *S. cerevisiae*, *FUR4* (uracil permease), *FUI1* (uridine permease) and *FCY2* (cytidine and cytosine permease), which belong to a large family of microbial purine-related transporters (PRT) (de Koning and Diallinas, 2000). The *FUR4* subfamily has 24 highly conserved amino acid residues, while the *FCY2* subfamily has ten. Of these ten, only one (N374) is conserved in *FCY2*. This asparagine residue is located at the cell surface and involved in substrate recognition (Bloch et al., 1992). The uracil permease is present in the cell membrane at very low concentrations and the *FUR4* mRNA has a half-life of 2 min (Chevallier, 1982). Upon arrival at the plasma membrane the protein gets phosphorylated, and in exponentially growing cells the Fur4p is rather stable (Volland et al., 1992; Volland et al., 1994). Under adverse conditions (high temperature, nutrient starvation), Fur4p is rapidly degraded, but also an increase in the internal uracil concentration has the same effect (Seron et al., 1999; Volland et al., 1994). The degradation pathway of Fur4p has been studied intensely, and it involves ubiquitination, endocytosis and subsequent proteolysis in the vacuole (Blondel et al., 2004; Galan et al., 1994; Galan et al., 1996; Marchal et al., 2000; Volland et al., 1994). Recently two uracil transporters (UPS1 and UPS2) from the plant, *Arabidopsis thaliana*, were characterized (Schmidt et al., 2004), but no other pyrimidine transporters from higher eukaryotes have been identified.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>FUR1</em></td>
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<td>JUND 1970</td>
</tr>
<tr>
<td><em>FUR4</em></td>
<td>uracil transporter</td>
<td>CHEVALLIER 1982</td>
</tr>
<tr>
<td><em>FUI1</em></td>
<td>uridine transporter</td>
<td>WAGNER 1998</td>
</tr>
<tr>
<td><em>URK1</em></td>
<td>uridine/cytidine kinase</td>
<td>WAGNER 1998</td>
</tr>
<tr>
<td><em>URH1</em></td>
<td>uridine/cytidine ribohydrolase</td>
<td>KURTZ 2002</td>
</tr>
<tr>
<td><em>FCY1</em></td>
<td>cytosine deaminase</td>
<td>JUND 1970</td>
</tr>
<tr>
<td><em>FCY2</em></td>
<td>purine/cytosine/cytidine transporter</td>
<td>CHEVALLIER 1975</td>
</tr>
<tr>
<td><em>CDD1</em></td>
<td>cytidine deaminase</td>
<td>KURTZ 1999</td>
</tr>
</tbody>
</table>

Table 1: Pyrimidine salvage genes and gene products in *S. cerevisiae*. 
REDUCTIVE CATABOLISM OF PYRIMIDINES

The reductive pathway (Figure 4) starts with the reduction of the pyrimidine ring of uracil or thymine (but not cytosine) at the C5-C6 bond yielding either dihydrouracil (DHU) or D-dihydrothymine (DHT). This is done by the NAD⁺/NADP⁺-dependent dihydropyrimidine dehydrogenase (DHPDH, EC 1.3.1.1/1.3.1.2, respectively). The next step is hydrolytic cleavage of the dihydropyrimidine ring at the N3-C4 bond by dihydropyrimidine amidohydrolase (DHP, EC 3.5.2.2). The products are beta-ureidopropionate (BUP) or D-beta-ureidoisobutyrate (DBUIB), respectively.

Figure 4: Reductive pyrimidine degradation. First a NAD⁺/NADP⁺-dependent reduction is carried out by DHPDH. Then a hydrolysis of the dihydropyrimidines is done by DHP, and lastly a hydrolysis of the beta-ureido group by UP, results in ammonia, CO₂ and the beta-amino acids, DBAIB and BAL.
Third step is also an amidohydrolytic reaction carried out by beta-ureidopropionase (UP, EC 3.5.1.6), which produces ammonia, carbondioxide and beta-alanine (BAL) or D-beta-aminosobutyrate (DBAIB) respectively. The degradation pathway is said to stop at this level, but two more steps are involved in the full metabolism of the six pyrimidine atoms. The last nitrogen is removed from BAL and DBAIB inside the mitochondrial matrix by pyridoxal 5’-phosphate (PLP) dependent aminotransferases, producing malonic semialdehyde (MSA) and methylmalonic semialdehyde (MMSA), respectively. These become activated by coupling to coenzymeA (CoA) as acetyl-CoA and propionyl-CoA. This will be presented in more detail later. In humans the pyrimidine nucleosides are cleaved by thymidine phosphorylase (TP) and uridine phosphorylase (URP) to free pyrimidine bases and ribose-1’-monophosphate, and then the free pyrimidine base is degraded (Johansson, 2003).

**Dihydropyrimidine dehydrogenase**

The enzyme activity has been identified in a large number of different organisms, but it is the mammalian enzyme that is the most well-studied. Rat and pig liver enzymes have been purified, and found to be homodimers of 220 kDa and 206 kDa, respectively (Podschun et al., 1989; Shiotani and Weber, 1981). They are both NADPH dependent enzymes containing both flavin molecules and iron-sulfur clusters. The pig DHPDH has been suggested to be following a two-site ping-pong mechanism (Podschun et al., 1990). First flavin molecules in site 1 gets reduced by electrons from NADPH, which then leaves as NADP⁺, then the electrons are transferred via the iron-sulfur clusters to flavin at site 2, where uracil binds and gets reduced to DHU and then leaves. The Kₘ values were 10 ± 1 and 1.1 ± 0.1 µM for NADPH and uracil, respectively. Cloning of the pig and human DHPDH cDNA, showed that the enzymes consists of 1025 amino acids with calculated Mw of 111416 and 111398 dalton, respectively (Yokota et al., 1994). The sequences revealed (based on homology to known motifs) the locations of the different motifs, but with the crystal structure of pig DHPDH it has been possible to pinpoint the residues in much more details (Dobritzsch et al., 2001; Dobritzsch et al., 2002; Schnackerz et al., 2004). The promoter region of the human DHPDH gene has been characterized, and two regulatory elements located in the -121/+7 upstream region (Shestopal et al., 2000).
The only bacterial DHPDH that has been successfully purified and characterized is from *Alcaligenes eutrophus* (Schmitt et al., 1996). The native enzyme was termed a homotetramer with a molecular mass of approx 210 kDa, even though SDS-PAGE showed two subunits sizes (47.5 kDa and 52 kDa) with different N-terminal sequences. From *Brevibacillus agri* strain NCHU1002 the first pyrimidine degrading gene cluster was reported, containing three closely spaced genes designated *pydABC* encoding putative DHPDH, DHP and UP, respectively (Kao and Hsu, 2003). Recombinant DHP and UP were characterized, but the DHPDH, could not be purified, and no activity could be measured. It was discovered that pyrimidines does not serve as a sole nitrogen source, while DHU, BUP and BAL do. Apparently it is only DHP and UP which are transcribed as a polycistronic mRNA, and the transcript is induced by dihydrouracil specifically. This type of regulation has also been shown in the yeast *Saccharomyces kluyveri*, where *PYD2* and *PYD3* mRNA is induced by DHU, and to some degree BUP, but not uracil (Gojkovic et al., 2000; Gojkovic et al., 2001).

**Dihydropyrimidinase**

This zinc-dependent DHP has been purified to homogeneity from bovine, calf, pig and rat liver (Brooks et al., 1983; Jahnke et al., 1993; Kautz and Schnackerz, 1989; Kikugawa et al., 1994). They are homotetrameric proteins with monomer sizes of 56.5 kDa for bovine, and 54 kDa for the remaining three. The natural substrates for calf liver DHP are DHU and DHT, but glutarimide, thiohydantoin and barbiturate could also be hydrolyzed at 20 %, 1 % and 0.02 % of the DHT velocity, respectively. Diethylbarbiturate or DHO could be used as substrate or inhibit the enzyme. The forward reaction has a pH optimum between 8-10 with approx. 20 % of maximum $V_{\text{max}}/K_m$ at pH 6. This pH dependency could indicate the presence of a charged amino acid active in substrate binding and/or catalysis (Kautz and Schnackerz, 1989).

The genes encoding DHP’s have been cloned from mammals (Hamajima et al., 1996; Matsuda et al., 1996), nematodes (Li et al., 1992), insects and molds (Gojkovic et al., 2003) and yeast (Gojkovic et al., 2000). The proteins show high sequence homology with other zinc-dependent cyclic amidohydrolases like DHOT, hydantoinase and allantoinase, and a common ancestry has been proposed (Gojkovic et al., 2003; Kim and Kim, 1998). Structures of yeast (*S. kluyveri*) and slime mold (*D. discoideum*) DHP’s have been resolved recently (Lohkamp et al., 2006). They show the same
overall structure and active site architecture, as members of the amidohydrolase family (DHOT, hydantoinase). Since DHOT is a pyrimidine biosynthetic enzyme, the previously found sequence homology and now the finding of almost identical structural fold, strongly suggest a common origin of catabolic (DHP) and anabolic (DHOT) enzymes in pyrimidine metabolism.

In vertebrates, a group of DHP homologous proteins (DRP), is involved in both central nervous system and enteric nervous system (Horiuchi et al., 2000; Inagaki et al., 2000). It seems as if DRP’s are required in most cell tissues (especially the brain, heart and skeletal muscle), while DHP is confined to the liver and kidney (Hamajima et al., 1996).

**beta-Ureidopropionase**

The final catabolic enzyme, UP, has been purified to homogeneity from rat and calf liver (Tamaki et al., 1987c; Waldmann et al., 2005), and partly purified from maize (Walsh et al., 2001), mouse liver (Sanno et al., 1970), *Clostridium uracilium* (Campbell, 1960). Genes have been cloned from rat (Kvalnes-Krick and Traut, 1993), human (Vreken et al., 1999), *A. thaliana* (Walsh et al., 2001) and *S. kluyveri* (Gojkovic et al., 2001). Also putative UP encoding genes from *D. melanogaster* and *D. discoideum*, have been identified by heterologues expression in a UP (pyd3) deficient *S. kluyveri* strain (Gojkovic et al., 2001). Phylogenetic analysis of different UP’s divides them into two groups, one having *S. kluyveri* UP grouped with bacterial N-carbamoyl-L-amino acid amidohydrolases, and another with all other UP’s (Gojkovic et al., 2001). Bacterial N-carbamoyl-D-amino acid amidohydrolases were placed somewhere between the two UP groups, indicating that the yeast UP evolved independently from the UP from higher eukaryotes. The recently determined structure suggests that the yeast UP shares the origin with some proteases (Lundgren et al., 2003).

**OXIDATIVE CATABOLISM OF PYRIMIDINES**

The presence of the oxidative pathway was originally found in soil-bacteria belonging to the *Mycobacterium*, *Corynebacterium* and *Norcadia* genera (Hayaishi and Kornberg, 1952; Lara, 1952a; Lara, 1952b; Wang and Lampen, 1952). It was first
believed to be a 2-step pathway with uracil dehydrogenase (UDH, EC 1.1.99.19) converting uracil/thymine into barbiturate/5-methylbarbiturate. Then the barbiturates were hydrolyzed by barbiturase (BAA, EC 3.5.2.1) into urea and malonate/methylmalonate. It was first 50 years later when BAA was characterized from *Rhodococcus erythropolis*, it was clear that another enzyme was needed in order to change barbiturate into urea and malonate (Soong et al., 2001). The third enzyme was ureidomalonase (UM), which hydrolyzes ureidomalonate (product of the BAA reaction) to urea and malonate (Soong et al., 2002).

The UDH gene has not been found yet, but the enzyme was partly purified and it was found to be dependent on methylene blue as electron acceptor (Hayaishi and Kornberg, 1952).

BAA is a tetramer, with a theoretical monomer size of 39 kDa. It has the classical zinc-binding motif (D-X-H-X-H) known from the zinc-containing amidohydrolase superfamily, but where other members have the motif in the N-terminal part, barbiturase has it in the C-terminal part. Its closest relative is cyanuric amidohydrolase (CAA), which is involved in degradation of s-triazine herbicides (Karns, 1999). A notable thing is that CAA does not contain the two crucial histidines in the zinc-binding motif. It is speculated that the enzyme evolved from BAA, because of the unnatural substrate and its locations on a transposable element (Eaton and Karns, 1991).

**METABOLISM OF BETA-ALANINE AND D-AMINOISOBYTURATE**

In microorganisms, a major function of BAL is as a constituent of pantothenate biosynthetic pathway (Webb et al., 2004). Pantothenate is formed by condensation of (R)-pantoate, derived from valine biosynthesis, and BAL (Figure 4).
Since pantothenate (vitamin B\textsubscript{5}) is an essential constituent in animal nutrition, BALs must have a different function, than pantothenate precursor, in animals. BAL is found in the rat central nervous system (CNS) along with known neurotransmitter gamma-aminobutyrate (GABA) (DeFeudis and Martin, 1977). BAL has been shown to activate both glycine and GABA\textsubscript{A} receptors, and it works as a potent inhibitor of GABA uptake system in glial cells (Mabjeesh et al., 1992; Wu et al., 1993). Uptake systems in mouse brain and rat astrocytes have been demonstrated (Holopainen and Kontro, 1986; Kontro, 1983). The transport across the blood-brain barrier is mediated by a Na\textsuperscript{+}/Cl\textsuperscript{-} dependent mechanism (Komura et al., 1996). This indicates a role of BAL in the vertebrate CNS, and some of the neurological symptoms seen in patients with defects in uracil degradation has been attributed to a decline in BAL concentrations (van Kuilenburg et al., 1999a). Recently this view has been weakened by the finding of normal levels of BAL in cerebrospinal fluid (CSF) and only weakly lowered levels in urine and plasma of patients with DHPDH deficiency (first step in pyrimidine degradation) (Fiumara et al., 2003; van Kuilenburg et al., 2004). How the BAL homeostasis is kept in these patients is not known, but metabolism of BAL containing dipeptides like carnosine (beta-alanyl-histidine) and anserine (beta-alanyl-1-methyl-histidine) might play a role. In Drosophila melanogaster, BAL is involved in pigmentation, by condensation with dopamine to form N-beta-alanyl-dopamine, which in turn gets oxidized yielding a tan pigment. A mutation leading to a black
pigmentation (black, b), was shown to be caused by hyperactivity of BAL catabolizing enzyme, and a suppressor mutation (su(b)) was found to have decreased activity (Weber et al., 1992). BAL has also been shown as a neurotransmitter in insects (Sandberg and Jacobson, 1981).

The function of DBAIB, the corresponding product originating from thymine degradation, is largely unknown, but also this molecule is thought to have a function in the CNS. Patients having DHPDH deficiency, show, contrary to BAL, a decrease in DBAIB level in CSF, and this might explain the neurological abnormalities seen in these patients (van Kuilenburg et al., 2004).

**Biosynthesis**

There are two major pathways for BAL synthesis besides uracil degradation, which were presented in the previous parts. These are L-aspartate decarboxylation and polyamine breakdown, but also other pathways have been proposed (Figure 5). In *E. coli*, direct decarboxylation of L-aspartate by the *panD* gene product, aspartate decarboxylase (ASPDC, EC 4.1.1.15) gives BAL and CO$_2$ (Cronan, Jr., 1980). The *panD* gene along with the three other *pan* genes, *panB* (ketopantoate hydroxymethyltransferase, EC 2.1.2.11), *panC* (pantoate-BAL ligase, EC 6.3.2.1), and *panE* (2-dehydropantoate 2-reductase, EC 1.1.1.169) constitutes the pantothenate biosynthesis pathway. Figure 5: Different ways of BAL synthesis. The major routes are the prokaryotic decarboxylation of L-aspartate, the yeast breakdown of putrescine, spermine and spermidine, and the more widely distributed reductive degradation of uracil found in bacteria, yeast, insects and mammals. The minor routes are hydrolysis of the dipeptides carnosine and anserine, and the multi-step conversion of propionate to BAL with malonic semialdehyde being the immediate precursor.
biosynthetic pathway. In *S. cerevisiae*, the *ECM31* and *PAN6* (*YIL145c*) genes are homologous to *panB* and *panC*, respectively, and are required for pantothenate biosynthesis (White et al., 2001). *PAN5* (*YHR063c*) is a structural homolog of *panE*, and is therefore thought to be involved in (R)-pantoate synthesis. Instead of using aspartate as a BAL source, *S. cerevisiae* uses two specialized aldehyde dehydrogenases (*ALD2* and *ALD3*) to convert 3-aminopropanal to BAL (White et al., 2003). The 3-aminopropanal is synthesized by polyamine degradation (from three *SPE* genes) and the amine oxidase encoded by *FMS1* (White et al., 2001). In some yeast, like *A. nidulans* and *S. pombe*, BAL (for pantothenate synthesis) is derived from uracil degradation and not polyamine breakdown or aspartate decarboxylation (Arst, Jr., 1978; Stolz et al., 2004). Uracil and beta-ureidopropionate (BUP), but not dihydrouracil (DHU) could serve as a BAL source in *S. pombe*. It was proposed that uracil is converted to BAL in an alternative way, bypassing DHU and maybe also BUP (Stolz et al., 2004). The *pantoC-3* mutant of *A. nidulans* could use BUP, but not uracil or DHU as BAL source and was believed to be blocked in DHP (second step of uracil degradation) (Arst, Jr., 1978). It was also found that 10 mM DL-beta-aminoisobutyrate ([DL]BAIB) could be used as a BAL source in the *pantoC-3* strain, but not by a *pantoC-3* *gatA-2* (gamma-aminobutyrate aminotransferase, GABA-AT). This led to the conclusion, that a GABA-AT catalyzed conversion of malonic semialdehyde could serve as a BAL source.

### Catabolism

BAL and DBAIB are further transported into the mitochondria where they are catabolized to malonic semialdehyde (MSA) and methylmalonic semialdehyde (MMSA), respectively (Mizota et al., 1988; Tamaki et al., 1987b). In mammals this is done by BAL aminotransferase (BAL-AT, EC 2.6.1.19) and DBAIB aminotransferase (DBAIB-AT, EC 2.6.1.40), respectively (Tamaki et al., 1982; Ueno et al., 1990). There is an enormous substrate overlap within this group of enzymes (Table 2), which has resulted in some enzymes were given multiple names.
Chapter 2

Table 2: Relative activities of purified BAL-AT and DBAIB-AT. Numbers are as percentage of the enzyme activity. The highest AMINO DONOR and AMINO ACCEPTOR activity for each enzyme is set to 100. N.D. = not determined.

<table>
<thead>
<tr>
<th>AMINO DONOR</th>
<th>BAL-AT (Rabbit)</th>
<th>BAL-AT (Rat)</th>
<th>DBAIB-AT (Rat)</th>
<th>DBAIB-AT (Rat)</th>
<th>BAL-AT (B.cereus)</th>
<th>GABA-AT (B.cereus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Alanine</td>
<td>76</td>
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<td>60</td>
<td>100</td>
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<tr>
<td>γ-Aminobutyrate</td>
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<td>1</td>
<td>43</td>
<td>100</td>
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<tr>
<td>δ-Aminovalerate</td>
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<td>95</td>
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<td>80</td>
</tr>
<tr>
<td>DL-Aminoisobutyrate</td>
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<td>48</td>
<td>78</td>
<td>48</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D-Aminoisobutyrate</td>
<td>N.D.</td>
<td>1</td>
<td>100</td>
<td>100</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>L-Aminoisobutyrate</td>
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<td>65</td>
<td>0</td>
<td>14</td>
<td>N.D.</td>
<td>N.D.</td>
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<table>
<thead>
<tr>
<th>AMINO ACCEPTOR</th>
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<th>Glyoxylate</th>
<th>Oxaloacetate</th>
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<tbody>
<tr>
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<tr>
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<td>27</td>
<td>6</td>
<td>33</td>
<td>5</td>
</tr>
</tbody>
</table>

* (Tamaki et al., 1982), † (Fujimoto et al., 1988), † (Tamaki et al., 1990), † (Ueno et al., 1990), † (Yonaha et al., 1985)

For example the enzyme L-beta-aminoisobutyrate aminotransferase (LBAIB-AT, EC 2.6.1.22), which is involved in valine metabolism, and GABA aminotransferase (GABA-AT, EC 2.6.1.19) are identical to BAL-AT (Kontani et al., 1999; Tamaki et al., 1987a), and alanine-glyoxylate aminotransferase 2 (AGT 2, EC 3.6.1.44), is identical to DBAIB-AT (Kontani et al., 1993). Because both BAL-AT and DBAIB-AT can use BAL as donor, they are sometimes termed BAL-AT I and BAL-AT II, respectively, where the real difference is that BAL-AT I specifically uses αKG as acceptor (EC 2.6.1.19) and BAL-AT II uses pyruvate (EC 2.6.1.18). It was found that in rats, BAL-AT I was the sole activity present in the brain, and in the liver and kidney it was seven times higher than BAL-AT II (Kontani et al., 1999). This identifies BAL-AT I as the major BAL catabolizing enzyme in mammalian systems, and BAL-AT II should be called DBAIB-AT. Since some bacterial BAL-AT enzymes, which use pyruvate as acceptor, cannot use [DL]BAIB as donor, these are indeed true BAL-AT II enzymes. To reduce confusion a list of the names with indication of the difference between them based on their activities is shown in Table 3. The names listed in this table will be used throughout the text. Both brain and liver BAL-AT I are localized in the mitochondrial matrix (Schousboe et al., 1977; Tamaki et al., 1987b).
The rat brain and liver type BAL-AT I differ in the N-terminal amino acid sequence, both to each other, but also to the predicted sequence from rat cDNA, but the activity of the two enzymes were practically the same, only a little difference in $K_M$ for BAL was seen (Kontani et al., 1999). The difference in the N-terminal is due to the proteolytic activities of the two mitochondrial endopeptidases, which produces either the mature brain BAL-AT I or the mature liver BAL-AT I. The processing protease from the rat liver was identified as the 418-1305 peptide of carbamoylphosphate synthetase I (Ohyama et al., 2004). The human BAL-AT I gene is highly expressed in brain, liver, kidney and pancreas (Jeon et al., 2000).

The products of the BAL-AT I and DBAIB-AT reactions are, as mentioned before, MSA and MMSA. These compounds are further metabolized to acetyl-CoA and propionyl-CoA by MMSA dehydrogenase (MMSADH) (Goodwin et al., 1989). In rats the enzyme is found in kidney and liver tissue, while neither mRNA or protein can be detected in the brain (Kedishvili et al., 1992). This distribution is much different from the BAL-AT I (liver, kidney and brain), but the same as DBAIB-AT (liver and kidney), as mentioned in previous section. This raises a question on how the rat catabolizes BAL in the brain, and if that does not happen, why is there BAL-AT I activity in the brain?. Either there is a specific brain type MMSADH or GABA is totally dominating the enzyme, hereby preventing BAL/DBAIB degradation. Because of MMSADH involvement in valine degradation, this enzyme has also been characterized and even crystallized from bacteria (Dubourg et al., 2004; Zhang et al., 1996).

Most yeast have the ability to utilize BAL as a sole nitrogen source (LaRue and Spencer, 1968). Usually yeast have a BAL-AT II and a GABA-AT (Yonaha et al., 1983). In *S. cerevisiae* only the GABA-AT is present, and BAL cannot be degraded in this organism. In *A. nidulans* and *U. maydis* mutation in the *gatA* and *ugatA* loci respectively, decrease the ability to utilize BAL as sole nitrogen source, indicating

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Table 3: Terminology used for BAL degrading enzymes.

<table>
<thead>
<tr>
<th>Name</th>
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<tr>
<td></td>
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<tr>
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<td>-</td>
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<tr>
<td>BAL-AT II</td>
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<tr>
<td>DBAIB-AT</td>
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<tr>
<td>GABA-AT</td>
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<td>+/-</td>
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</table>
Chapter 2

that these loci might encode BAL-AT I enzymes. The fact that they still grow to some
degree, despite the presence of a mutation, shows that there is a substrate overlap with
other aminotransferases in the cell. Nothing is known on MSA or MMSA metabolism
in yeast.

CLINICAL ASPECTS OF DEFECTS IN PYRIMIDINE
DEGRADATION IN MAN

Genetic deficiencies

Inborn errors in the all three enzymes of the pyrimidine catabolic pathway have been
identified (Berger et al., 1984; Duran et al., 1991; Moolenaar et al., 2001). The most
commonly encountered is the DHPDH deficiency (more than 50 cases), while both
the DHP deficiency and the UP deficiency has been described in around 5-10 patients
each. The most common genotype leading to DHPDH deficiency is the IVS14+1G>A
(> 50% of patients), which leads to a deletion of a 165-bp fragment (van Kuilenburg
et al., 1999a). Usually, patients have first been diagnosed for having motor retardation
and mental retardation for some time (Christensen et al., 1998; van Kuilenburg et al.,
1999b; Vreken et al., 1998). Urinary, plasma and CSF levels of thymine and uracil are
elevated in DHPDH patients. Neurological abnormalities in patients suffering from
DHPDH, DHP and UP deficiency have been explained by lowered BAL
concentrations caused by the block in pyrimidine degradation. A study of the BAL
and DBAIB concentrations in DHPDH patients, showed that BAL homoestasis was
intact, indicating an alternative route for BAL synthesis (van Kuilenburg et al., 2004).
DBAIB was significantly lower in DHPDH patients compared to controls, which
might suggest that some of the abnormalities seen in patients, could originate from
altered DBAIB instead. Nothing is known on the BAL and DBAIB homoestasis in
patients with DHP and UP deficiency, since patients have not been tested for
compounds or enzyme activities after the identified block. In patients with UP
deficiency (UP activity is absent), a large increase in urinary BUP and DBUIB is
detected (van Kuilenburg et al., 2001). Pyrimidine and dihydropyrimidine
concentrations are slightly increased. A possible role of BUP in the neuropathology of
patients with UP deficiency and patients with severe propionic aciduria has been
proposed (Kolker et al., 2001). Deficiency in BAL-AT I is also very rare. The patients
have seizures, brain abnormalities and a high-pitched cry, an index of severe CNS disease (Medina-Kauwe et al., 1999). A large increase in GABA, homo-carnosine and BAL concentrations in both plasma and CSF was seen. The severity of the BAL-AT I disease, must be ascribed to the abnormal GABA metabolism, rather than the BAL metabolism. The first case of MMSADH deficiency was found in 1981 (Congdon et al., 1981). A characteristic over-excretion of BAL, [DL]BAIB and [DL]-beta-hydroxyisobutyrate, along with an impairment in oxidation of 2-C of valine and 1-C of BAL (Gray et al., 1987; Pollitt et al., 1985). In another case normal BAL and beta-hydroxypropionate excretions was seen, but valine and thymine metabolism showed clearly a deficiency in MMSADH (Roe et al., 1998). While the former patient was perfectly healthy, the latter showed signs of developmental delay, but still because of the mild phenotype, patients are rarely found.

**Pharmacological influence**

A lot of cancer types like colorectal, breast and head and neck are treated with the chemoterapeutic agent 5-fluorouracil (5-FU) (van Kuilenburg, 2004). The 5-FU needs to anabolised to the nucleotide level in the cell in order to exert its cytotoxicity. It gets incorporated into RNA as 5-fluorouridine 5’-triphosphate (5-FUTP) and into DNA as 5-fluoro-2’-deoxyuridine 5’-triphosphate (5-FdUTP) leading to destabilization of both. While these effects on the nucleic acid stability should be devastating for the cells, it is believed that the most profound anti-tumour effect exerted by 5-FU arises when it has been anabolised to the 5-fluoro-2’deoxyuridine 5’-monophosphate (5-FdUMP) level. 5-FdUMP is a potent inhibitor the enzyme thymidylate synthase (TS), which is responsible for the methylation of dUMP. The dosage and administration schedule of the drug needs to be carefully planned in order to minimize the side effects from the treatment. DHPDH is believed to be a key determinant in the toxicity of 5-FU, while heterozygotes in DHP and UP do not seem to be affected. The reductive degradation of 5-FU leads to alpha-fluoro-beta-alanine (FBAL) and it’s the major (> 95 %) of urinary catabolites (Diasio and Harris, 1989). It has been found that defluorination of FBAL is caused be BAL-AT II in rat liver homogenates (Porter et al., 1995). The natural substrates of DHP is six-membered pyrimidine rings (eg. DHU and DHT), but it can also hydrolyze drugs based on the five-membered rings hydantoin and succinimide (Dudley et al., 1974).
INTRODUCTION TO YEAST

Yeast have been used for millenia as “producers” of beer, wine and bread (Piskur et al., 2006), and recently also as producers bio-ethanol, vitamins and pharmaceutical products like hormones and protein drugs, through heterologues expression. Despite these ”good” purposes several yeast species are pathogenic to e.g. humans and plants. The most well-known yeast is *Saccharomyces cerevisiae*, which offers unique opportunities to study eukaroytic gene regulation and evolution, cell cycle, metabolic pathways and other molecular genetics and cell biology related subjects. The many years of focus on *S. cerevisiae* has left its genome thoroughly annotated, and even a functional profiling of the genome has been made (Giaever et al., 2002). Because of these effort’s in making *S. cerevisiae* the top yeast model organism, it is often used as a reference for annotations of genes from other organisms. In the recent years a number of genomic sequencing projects has been undertaken and now at least 16 annotated fungal genomes are collectively available in NCBI databases. The power of having more genomes sequenced, is e.g. the annotation of ORF’s can be done more easily, if other homologs can be found. Start and stop codons are better determined, if the size of homologous proteins in other organisms is known. Comparative genomic analysis has greatly redefined the *S. cerevisiae* proteome, since comparisons of closely related species, reveal wrongly annotated genes. It is estimated that approx. 500 of approx. 6000 annotated genes should be eliminated, approx. 300 start or stop codons should be changed (Kellis et al., 2003). Especially the non-coding regions of the genome are getting a lot of attention, since intergenic functional elements are difficult to find from a single genome sequence of poorly studied yeasts (Cliften et al., 2001; Kellis et al., 2003).

Phylogeny

For a long time classification of yeast species in genera and families were based on morphology, sexual states and physiology. With the bioinformatical approach, based on sequences from slowly evolving genes like ribosomal DNA, the former classification has been redefined a number of times. Recently Kurtzman et al. divided the "*Saccharomyces* complex” (Saccharomyces related species) into 14 clades (Kurtzman and Robnett, 2003). The resulting tree clearly showed that the previous
division of the species into taxa, based on behavior and abilities (phenotype), were not supported by their DNA sequence relationship (genotype). A simplified phylogenetic tree of the "Saccharomyces complex" is presented in Figure 6. Especially two genera are split, as mentioned in the figure caption. Strains from the Saccharomyces genus has been divided into three groups; sensu stricto, sensu lato and an outgroup composed by S. kluyveri (Barnett, 1992). This would translate into groups, where sensu stricto species belong to clade 1, sensu lato species belong to clade 2 and 3, while S. kluyveri as an outgroup belong to clade 10.

![Figure 6: Simplified phylogenetic tree adapted from Kurtzman and Robnett, 2003. Species from each of the 14 clades (branch points) are presented with S. cerevisiae being clade 1. It is seen that the genera Kluyveromyces (underlined) are found in two groups one close to S. cerevisiae (Clade 2, 4, 5, 6) and one distant (Clade 10, 11). In Clade 10 is also found a Saccharomyces yeast, namely S. kluyveri (bold).](image)

**Nitrogen metabolism and regulation**

The flow of nitrogen is a central metabolic entity in microorganisms. Different yeast can utilize a variety of different compounds as sole nitrogen sources, indicating the presence of different specific catabolic pathways (Large, 1986). In general nitrogenous compounds like amino acids are easily utilized through transaminase reactions leading to glutamate, which is the predominant amino donor in many biosynthetic reactions. If a compound can serve as a nitrogen source, then usually all
intermediates in the conversion from the compound to nitrogen (ammonia or glutamate) can be used. This is of course dependent on effective transport systems for the intermediates. An example of this is the *S. cerevisiae* allantoin degradation pathway. Allantoin is a degradation product from purine degradation, and its further degradation goes through five steps, before all four nitrogen atoms are liberated as ammonia (Figure 7). The first three steps are dependent on the *DAL1-3* genes, encoding allantoinase (EC 3.5.2.5), allantoicase and ureidoglycolate hydrolase, respectively (Buckholz and Cooper, 1991; Yoo et al., 1985). This results in production of two urea molecules. *S. cerevisiae* does not have the normal urease (EC 3.5.1.5.), but instead urea is degraded by the *DUR1,2* gene product, a multifunctional urea amidolyase and allophanate hydrolase (Cooper et al., 1980).

![Allantoin degradation](image)

*Figure 7: Allantoin degradation. Dal1p = allantoinase, Dal2p = allantoicase, Dal3p = ureidoglycolate hydrolase, Dur1p = urea carboxylase, Dur2p = allophanate hydrolase.*

The genes in allantoin pathway are induced by the end-product allophanate or a non-metabolizable analog oxalurate (Cooper and Lawther, 1973; Sumrada and Cooper, 1974). Another pathway is the gamma-aminobutyrate (GABA) pathway (Ramos et al., 1985). The catabolism of GABA is performed by *UGA1* and *UGA2*, encoding GABA-AT and succinic semialdehyde dehydrogenase (SSADH, EC 1.2.1.16). This pathway
is induced by GABA, and regulated by the \textit{UGA3} gene product (Andre and Jauniaux, 1990).

The nitrogen metabolism are controlled on two levels; global (via nitrogen catabolite repression, NCR) and pathway (via specific inducers). Two GATA-family transcription factors Gat1p and Gln3p, function as global activators of for example the \textit{DUR}, \textit{DAL} and \textit{UGA}, when a poor nitrogen source (proline) is present. When a good nitrogen source (glutamine, asparagine or ammonia) becomes available, Gat1p and Gln3p gets phosphorylated, excluded from the nucleus and prevented from reentering by the Ure2 protein (Cox et al., 2000; Cunningham et al., 2000a). Two other GATA-family transcription factors Dal80p and Deh1p function as global repressors by competing with Gat1p and Gln3p (Coffman et al., 1997; Cunningham et al., 2000b; Svetlov and Cooper, 1998). Two non-global activators, Dal81p and Dal82p are involved in inducer specific activation of different pathways (Bricmont et al., 1991; ElBerry et al., 1993). The pathway specific inducer Uga3p, has only been found to induce \textit{UGA} genes (Andre, 1990).

\textbf{Pyrimidine degradation in yeast/fungi}

It was Di Carlo et al that in 1952 made the first real study of pyrimidine degradation in yeast (Di Carlo et al., 1952). The reference was primarily work done on dogs by Cerecedo, Emerson and Stekol in the period from 1927-33 (Cerecedo, 1927; Cerecedo, 1930; Cerecedo, 1931; Emerson and Cerecedo, 1930; Stekol and Cerecedo, 1931; Stekol and Cerecedo, 1933). Cerecedo and co-workers had come to the conclusion that the sequence of pyrimidine breakdown in dogs, was uracil $\rightarrow$ isobarbiturate $\rightarrow$ isodialurate $\rightarrow$ urea + oxalic acid. Di Carlo and co-workers analyzed \textit{S. cerevisiae} and \textit{Torula utilis} (\textit{Pichia jadini}) for ability to grow on 64 different compounds. While the nitrogen from 14 compounds were fully assimilated by \textit{T. utilis} only 4 (asparagine, aspartate, DHO and oxalurate) were assimilated in \textit{S. cerevisiae}. A route leading from uracil $\rightarrow$ DHU $\rightarrow$ DHO $\rightarrow$ urea was finally suggested. Two important observations were made; thymine and barbiturate did not support the growth of either species, and among the assimilated compounds by \textit{T. utilis} uracil, DHU, BUP and BAL were found. Shortly after, the reductive degradation of both thymine and uracil was showed in rats, and the reversibility of the first and second step, but not the third was demonstrated (Fink et al., 1952; Fink et al., 1953; Fink et
al., 1956). In the filamentous fungus *Neurospora crassa* appearance of DHU and BUP was reported in media from cells grown with excess uridine, and both uracil, DHU and BUP, but not BAL was shown to complement a pyrimidine requiring mutant (Woodward et al., 1957). Now, it looked as if the pyrimidine catabolic pathway of mammals and fungi, were at least very similar if not identical. This hypothesis was strengthened by new experiments on *Torulopsis utilis* (*P. jadinii*) demonstrating *in-vitro* DHP activity in uracil grown cells, and the observation that cells adapted fast to a change in media from uracil to DHU or BUP (Piret et al., 1964). But it did not take long before urea was again postulated as the end-product of uracil degradation in two basidiomycetes, *Agaricus bisporus* and *Lycoperdon pyriforme* (Reinbothe, 1964). This time without the involvement of DHU, but rather via an oxidative pathway not including barbiturate or a reductive pathway implying ribotide derivatives of pyrimidines.

Finally in 1968 a systematic (and ambitious) study on utilization of pyrimidines by 127 species of yeast was published (LaRue and Spencer, 1968). Cytosine, uracil, thymine, DHU, DHT, BAL and [DL]BAIB were tested as sole nitrogen sources, and the result was complex. The uracil pathway was the most abundant with 69, 53, 69 strains growing on uracil, DHU and BAL respectively. In the thymine pathway 13, 28, 79 strains grew on thymine, DHT and [DL]BAIB respectively. Of the 69 strains growing on uracil 48 grew on DHU, and of the 13 strains growing on thymine four grew on DHT. All in all, there was no clear evidence for a widespread intact reductive pathway within the yeast, but practically all non-*cerevisiae* like *Saccharomyces* strains tested, could grow on the tested compounds except thymine. However, the main deficiency of these studies was the phylogenetic relationship among the tested yeasts was not clear.

It was not until 1998, that the study of the yeast pathway, was taken up again, focusing on *S. kluyveri*, which contrary to indications from the name, is a not a close relative of *S. cerevisiae*. (Gojkovic et al., 1998). Here random whole genome mutagenesis was used combined with mutant screening on media containing uracil or BAL as sole nitrogen sources. Four classes of mutants were produced, designated *pyd1, pyd2, pyd3* and *bac* (PYrimidine Degradation and Beta-Alanine Catabolism) based on their lack of ability to use uracil, DHU, BUP or BAL as sole nitrogen sources. The reductive pathway has now been described in much detail in the yeast, *S.
**Introduction**

*Saccharomyces kluyveri*, Two of the three genes involved (*PYD2* and *PYD3*) have been identified by functional complementation of *pyd2* and *pyd3* mutants respectively (Gojkovic et al., 2000; Gojkovic et al., 2001). The two gene products have been crystalized (Dobritzsch et al., 2003; Dobritzsch et al., 2005) and the structure have been determined (Lohkamp et al., 2006; Lundgren et al., 2003). Even though these findings represent a strong evidence for the presence of a functional reductive pyrimidine catabolic pathway in yeast, the first gene (*PYD1*) has not been identified yet. The Pyd1p enzymatic activity (DHPDH), has never been proven *in-vitro*, but indirect *in-vivo* evidence of the activity was demonstrated when 120 times higher amount of BUP was excreted by *pyd3* cells compared to wild type cells when grown in proline + uracil media (both 0.1 %) (Gojkovic et al., 2001).

*Saccharomyces kluyveri*

It was first isolated from *Drosophila pinicola* in California (Phaff et al., 1956). It was found to be heterothallic, requiring two different mating types to sporulate (Wickerham, 1958). Sporulation results in 4 spherical spores per ascus, but the asci do not rupture upon maturity, which sometimes presents a problem in the laboratory. The first auxotrophic mutants described were *ura3* selected on 5-fluoroorotate (Fujimura, 1991). It was also shown that *S. kluyveri ura3* could be complemented, when transformed with a centromere-based plasmid carrying the *URA3* gene from *S. cerevisiae*. So both the stable replication of the centromere-based plasmid and the expression of a foreign gene was demonstrated. The *HIS3* gene of *S. kluyveri* was partially removed in a *ura3* background, showing the possibility to use homologous recombination as a molecular technique (Weinstock and Strathern, 1993). The *Saccharomyces* genus has been divided into petite-positive and petite-negative species, where the latter consists of only one, namely *S. kluyveri*. *S. kluyveri* can grow anaerobically, like all other *Saccharomyces* species, but it is not able to produce petites (Moller et al., 2001a). The ability to grow without oxygen, seems to be related to the *de novo* pyrimidine biosynthetic enzyme dihydroorotate dehydrogenase (DHODH) (Gojkovic et al., 2004). The anaerobic *S. cerevisiae* has a cytosolic oxygen independent DHODH (ctDHODH), while the aerobic, *Schizosaccharomyces pombe* has a mitochondrial oxygen dependent DHODH (mtDHODH). *S. kluyveri* seems to be in between in that it has both the ctDHODH and the mtDHODH, and therefore has its
pyrimidine biosynthesis uncoupled from the respiratory chain. Since it’s not enough to have this ctDHODH for *S. kluveri* to produce respiratory petites, other factors must be involved. The use of *S. kluveri* as a heterologues protein producer showed a 3.6 fold higher yield than a *S. cerevisiae* reference strain, when producing proteinase A (Moller et al., 2001b). What really makes *S. kluveri* unique in the *Saccharomyces* genus is its ability to degrade a wide variety of nucleobases (Gojkovic et al., 1998; LaRue and Spencer, 1968). Especially the pyrimidine degradation pathway has been studied, and it has been shown that *S. kluveri* degrades pyrimidines via the reductive pathway (described in more detail previously). The *S. kluveri PYD2* gene encoding DHP, was the first gene found to be involved in pyrimidine degradation in unicellular eukaryotes (Gojkovic et al., 2000). Because of its evolutionary placement between primitive oxygen dependent and modern oxygen independent yeast, further studies into this organism might give more detailed information on the mechanism that drive evolution in the yeast genera.

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CHAPTER 3

CATABOLISM OF PYRIMIDINES IN YEAST:
A TOOL TO UNDERSTAND DEGRADATION OF
ANTI-CANCER DRUGS*

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* This manuscript is in press (Nucleosides Nucleotides Nucleic Acids)
Catabolism of pyrimidines in yeast:  
a tool to understand degradation of anti-cancer drugs

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ABSTRACT

The pyrimidine catabolic pathway is of crucial importance in cancer patients, because it is involved in degradation of several chemotherapeutic drugs, as 5-fluorouracil, but it is also important in plants, unicellular eukaryotes and bacteria for the degradation of pyrimidine-based biocides/antibiotics. During the last decade we have developed a yeast species, *Saccharomyces kluyveri*, as a model and tool to study the genes and enzymes of the pyrimidine catabolic pathway. In this report we studied degradation of uracil and its putative degradation products in thirty-eight yeasts and showed that this pathway was present in the ancient yeasts but was lost app. 100 million years ago in the *S. cerevisiae* lineage.

Keywords: uracil degradation, pyrimidines, yeast, evolution, cancer.

INTRODUCTION

Pyrimidine bases, among them several anti-cancer drugs, are degraded via a reductive or an oxidative pathway (Vogels and van der Drift, 1976). The reductive pathway is better studied, primarily because it is important in humans. The enzymes involved in the reductive degradation have been purified from a variety of organisms, like mammals, insects, molds, yeast and bacteria (Gojkovic et al., 2003; Schmitt et al.,
Origin of pyrimidine degradation in yeast

1996; Yokota et al., 1994). The crystal structures of pig dihydropyrimidine dehydrogenase (DHPDH, EC 1.3.1.2), catalyzing the conversion of uracil to dihydrouracil (DHU) (Dobritzsch et al., 2001), and Saccharomyces kluyveri beta-ureidopropionase (UP, EC 3.5.1.6), catalyzing the conversion of beta-ureidopropionate (BUP) to beta-alanine (BAL) (Lundgren et al., 2003), have been solved. S. kluyveri dihydropyrimidinase (DHP, EC 3.5.2.2), which opens the DHU ring resulting in BUP has recently been crystallized (Dobritzsch et al., 2005). In addition, two S. kluyveri catabolic genes, PYD2 and PYD3, encoding DHP and UP, and their expression have been characterized in detail (Gojkovic et al., 2000; Gojkovic et al., 2001). Thereby, S. kluyveri, is becoming a useful model to understand the degradation of pyrimidines. However, the fate of uracil in yeast and the involved genes and enzymes are still largely unknown. In this paper, we would like to address the origin of the present situation regarding the pyrimidine catabolism in yeast. Thirty-eight yeast species belonging to the genera Saccharomyces, Arxiozyma, Kluyveromyces, Candida, Zygosaccharomyces, Torulaspora and Hanseniaspora were analyzed for their growth on uracil, DHU, BUP and BAL as the sole nitrogen source. One should keep in mind that the ability to utilize uracil as the sole nitrogen source is a complex of several biochemical pathways and the corresponding regulatory mechanisms.

MATERIALS AND METHODS

Strains
The strains used in growth experiments and their accession numbers are listed in Table 1. All strains are maintained in the ARS Culture Collection (NRRL), National Center for Agricultural Utilization Research (Peoria, Illinois USA), except Zygosaccharomyces bailii (ISA 1307) from Culture Collection of the Instituto Superior de Agronomia (Lisbon, Portugal), Zygosaccharomyces bailii (ATCC 36947) and Zygosaccharomyces bailii (ATCC 60483) from American Type Culture Collection (Manassas, Virginia USA) and Kluyveromyces lactis (CBS 2359) from Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands).
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Growth test
Uracil, DHU, BUP and BAL were purchased from Sigma. Yeast Nitrogen Base w/o amino acids and ammonium sulphate was purchased from Difco. The growth test was done by spotting 5 µL of culture on synthetic defined (SD) plates (1 % succinic acid, 0.6 % sodium hydroxide, 2 % glucose, 0.17 % yeast nitrogen base w/o amino acids and ammonium sulfate, 2 % agar) supplemented with 0.5 % ammonium sulfate (control) or 0.1 % uracil, DHU, BUP and BAL respectively. Growth was determined after 7 days at 30°C. All given percentages are in w/v.

RESULTS

Utilization of uracil, DHU, BUP and BAL
The ability of different strains to grow on uracil, DHU, BUP or BAL, as the sole nitrogen source, is shown in Table 1. The different species analyzed are listed according to their phylogenetic relationship, as reported by Kurtzman and Robnett (Kurtzman and Robnett, 2003). Note that the present yeast nomenclature does not reflect their phylogenetic relationship. For example, S. kluyveri is not very closely related to other Saccharomyces yeasts. In other words, higher a species is listed in this table, more closely it is related to S. cerevisiae. The growth was classified as no growth (-), some growth (+) and full growth (++), compared to the control plates (with ammonium sulfate as the sole nitrogen source). It is interesting to point out that the growth on uracil, DHU or BUP is in general linked in all species, but K. lodderae and H. valbyensis (Table 1).

Loss of pyrimidine catabolic pathway
Figure 1 shows a simplified tree of the Saccharomyces complex based on data from Kurtzmann and Robnett (2003) and summarizes the loss of the ability to grow on uracil, DHU, BUP and BAL. In general, the presence or absence of the tested abilities can be well explained as a function of the gene-loss events at various time-points in the evolutionary history. Uracil, DHU and BUP phenotypes are linked, and the ability to grow on these three compounds was “lost” independently and before the loss of the BAL phenotype (Figure 1). A few minor discrepancies are found within the ability to degrade BAL. The ability to utilize BAL was lost in the S. cerevisiae – S. rosinii
Origin of pyrimidine degradation in yeast

lineage (Table 1). Surprisingly, it is still found in *S. unisporus*, which is a very close relative of *S. servazzii*. *S. unisporus* has kept the ability even though it was lost in *A. telluris*, *S. spencerorum* and *S. rosinii*. Apparently, this ability has also, independently, been lost in the *K. delphensis* lineage (a close relative of *C. glabrata*) and *C. castellii*. *K. delphensis* and *C. castellii* has in fact lost this ability, even though all closely related species still posses it.

![Figure 1: The presence of pyrimidine degradation pathway. A simplified phylogenetic tree of five prominent yeast species is shown and the occurrence of the whole-genome duplication (WGD), which took place approximately 100 mill. years ago, is indicated. The ability to utilize uracil, DHU, BUP and BAL is shown next to the species.]

DISCUSSION

The yeast *S. kluyveri* can grow on uracil, DHU, BUP and BAL, which all are components of the reductive pyrimidine pathway known from humans, while *S. cerevisiae* cannot. The growth tests of thirty-eight strains from the *Saccharomyces* complex on uracil and the intermediates of the reductive pathway was done in order to understand the diversity and evolution of the ability to degrade pyrimidines. It seems that the ability to utilize uracil, DHU and BUP as sole nitrogen source was lost at approximately the same time, when the yeast genome was duplicated\(^\text{11}\), while the ability to use BAL was lost much later, and perhaps independently in a few lineages. Apparently, the major metabolic changes which followed the yeast genome duplication, made the possibility to regulate pyrimidine pools via degradation and to
produce BAL from BUP (for pantothenate synthesis) obsolete. The extensive sequencing of the yeast genomes (Piskur and Langkjaer, 2004) now provides a tool to find the genetic background for many phenotypes and to deduce their evolutionary history. However, one should keep in mind that we still do not understand the genetic

<table>
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<th>Strain (Accession number)</th>
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or biochemical background of uracil degradation in any yeast. In addition, the ability
to utilize uracil, DHU, BUP and BAL is a complex process which requires the
presence of the genes coding for the degradation enzymes, presence of the recipients
of the nitrogen originating from “poor” N-sources, an efficient uptake system, and a
complex regulatory net. When our knowledge on all these elements is improved in at
least one yeast species, a comparative genome analysis will add an even additional
insight on the evolution of the uracil, DHU, BUP and BAL degradation.

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structure of dihydropyrimidine dehydrogenase, a major determinant of the
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660.

5,6-dihydropyrimidine amidohydrolase, which participates in a novel fungal

Gojkovic, Z., Rislund, L., Andersen, B., Sandrini, M. P., Cook, P. F., Schnackerz, K.
D., and Piskur, J. (2003). Dihydropyrimidine amidohydrolases and
dihydroorotases share the same origin and several enzymatic properties.
Nucleic Acids Res. 31, 1683-1692.

are functionally related but have a high degree of structural diversity. Genetics
158, 999-1011.
Chapter 3


CHAPTER 4

GENETIC ANALYSIS OF URACIL DEGRADATION IN YEAST SACCHAROMYCES KLUYVERI:
THE DISCOVERY OF A “NOVEL” PATHWAY

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ABSTRACT

Have all metabolic pathways already been described? In mammals pyrimidine degradation starts with reduction of uracil to dihydrouracil (DHU), while in some bacteria uracil is initially oxidized to barbiturate. Previously, it has been shown that in the yeast Saccharomyces kluyveri, two loci, PYD2 and PYD3 are involved in degradation of DHU and beta-ureidopropionate (BUP). These two genes are a part of the reductive pathway of uracil degradation. However when the PYD2 and PYD3 genes were disrupted, the corresponding strains could still grow on uracil, but not on DHU or BUP. This suggested that in S. kluyveri uracil is not degraded via the reductive pathway. A mutagenesis approach and subsequent characterization of mutants revealed several genes (PYD11,12,13,14,15,16), that are involved in uracil degradation. Homology searches showed that these genes are also present in other fungi as well as in some bacteria, and in bacteria some of them (PYD11,14,16) are often clustered together. A HPLC method for separation of the reaction intermediates of the novel uracil degradation pathway was developed, and urea was identified as one of the intermediates along with five unidentified compounds. Apparently, the S. kluyveri PYD1 enzyme complex catalyzes a novel biochemical pathway, in which ribosylated uracil species (i.e. UMP) and urea seem to be the central intermediates.
INTRODUCTION

Previous literature reports that pyrimidine bases are degraded via a reductive or an oxidative pathway (Vogels and van der Drift, 1976). The reductive pathway, found in both eukaryotes and prokaryotes, consists of three enzymatic steps: dihydrouracil dehydrogenase (DHPDH), dihydropyrimidinase (DHP) and beta-ureidopropionase (UP). This pathway is most well studied in mammals, where substantial advances in understanding of its involvement in human disease (van Gennip et al., 1997) and pharmacokinetics of pyrimidine based drugs, like 5-fluorouracil (Kubota, 2003) have been achieved. The oxidative pathway has been found in soil bacteria of the genera, *Mycobacterium*, *Corynebacterium* and *Nocardia* (Hayaishi and Kornberg, 1952; Lara, 1952a; Lara, 1952b; Wang and Lampen, 1952), but in fact only one enzyme involved in oxidative degradation (barbiturase) has been purified so far (Soong et al., 2001). The corresponding gene has been sequenced and this enzyme has a very limited number of homologous proteins among the known sequences (Soong et al., 2002). Pyrimidine degradation has been examined in a variety of fungi, but no clear picture of the existing pathways has been found (Di Carlo et al., 1952; LaRue and Spencer, 1968; Reinbothe, 1964; Woodward et al., 1957). In recent years, the studies have been focused on the yeast, *Saccharomyces kluveri*, where a genetic approach, identified three genetic loci (*PYD1*, *PYD2*, *PYD3*) possibly involved in the degradation of uracil to beta-alanine (BAL) via dihydrouracil (DHU) and beta-ureidopropionate (BUP) (Gojkovic et al., 1998). The last two genes (*PYD2*, *PYD3*), have been characterized, and found to encode the yeast DHP (Pyd2p) and UP (Pyd3p) with low (28 %) or no identity to their human counterparts (Gojkovic et al., 2000; Gojkovic et al., 2001). Both *S. kluveri* DHP (Lohkamp et al., 2006) and UP (Lundgren et al., 2003) have been studied in detail, and their structure recently elucidated. However, so far the DHPDH activity (Figure 1), has not been measured in *S. kluveri*, and a homologous gene has not been identified within the *S. kluveri* genome sequence (Cliften et al., 2003).
In this paper six genetic loci, \textit{PYD11,12,13,14,15,16}, involved in the uracil degradation pathway in \textit{S. kluyveri} are presented. Homology searches indicate that the pathway is also found in other fungi and bacteria. In order to separate individual intermediates of the pathway, a HPLC method was developed, confirming that uracil degradation in \textit{S. kluyveri} represents a novel pathway. Speculations on the involvement of uracil degradation in beta-alanine (BAL) and pantothenate metabolism are discussed.

\section*{MATERIALS & METHODS}

\subsection*{Materials}
Uracil (U0570), DHU (D7628), BUP (94250) and BAL (05159) were purchased from Sigma. Yeast nitrogen base w/o amino acids and ammonium sulfate was purchased from Difco. \textit{[2-\textsuperscript{14}C]-uracil (MC124), [6-\textsuperscript{14}C]-uracil (MC159), [2-\textsuperscript{14}C]-uridine (MC105), [\textsuperscript{14}C]-urea (MC141), [2-\textsuperscript{14}C]-DHU (MC314), [carbamoyl-\textsuperscript{14}C]-BUP (MC2047), [\textsuperscript{3}H]-BAL (MT1527) were purchased from Moravek Biochemicals. Oligos used were purchased from DNA Technology, Denmark, and are presented in Table 1. Sequencing was done by MWG Biotech, Germany. }

\subsection*{Strains and growth media}
The \textit{S. kluyveri} strains used for the knock-out and mutagenesis studies, and the strains produced in these studies are presented in Table 2. The strains were grown in YPD medium (1 \% yeast extract, 2 \% Bactopeptone, 2 \% glucose) or synthetic defined (SD)/N-minimal medium (1 \% succinic acid, 0.6 \% sodium hydroxide, 2 \% glucose, 0.67 \% yeast nitrogen base w/o amino acids and ammonium sulfate) supplemented with different nitrogen sources (0.5 \% ammonium sulfate (SD), 0.1 \% for all other types (N-minimal)). For solid medium (plates) 2 \% agar was added. All percentages are w/v. The \textit{Escherichia coli} strain XL1-Blue (Stratagene) was used for plasmid
amplification. Bacteria were grown at 37°C in Luria-Bertani medium supplemented with 100 mg/L of ampicillin for selection (Sambrook et al., 1989). G418 selection media consisted of YPD supplemented with 75 mg/L of G418 (SIGMA G5013).

Table 1: Oligos used for targeted disruptions. Underlined sequences are reverse complements of either KanMX3 5’ or KanMX3 3’.

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Mutagenesis

Yeast mutants were generated from the strains Y156 and Y159 with ethyl methanesulfonate (EMS) as described by Gojkovic et al., 1998. Mutagenized cells
were plated on YPD plates (100-200 colonies per plate) and grown for 2-3 days at 25°C. The plates were replicated onto new SD or uracil N-minimal plates. After 5-7 days at 25°C, colonies were chosen based on inability to grow on uracil N-minimal plates compared to control plates. Strains were grouped based on interallelic complementation tests, which were carried out by crossing mutants on YPD plates and replica plating to uracil N-minimal plates (see Table 3).

**Complementation of pyd1 mutants with genomic library**

The *S. kluyveri* wild-type genomic library prepared by F. Lacroute, was based on the shuttle vector pFL44S (Bonneaud et al., 1991). Mutants from each of the complementation groups were transformed with the library DNA using electroporation procedure (Gojkovic et al., 2000) and plated on uracil N-minimal plates for selection. A number of transformants from each mutant strain were tested for plasmid loss, before rescue of the plasmid into the *E. coli* strain. Sequencing of the inserts was done using the primers M13rev-29 and M13uni-21 from MWG Biotech, Germany. The obtained sequences were searched for ORFs and the complementing ORF was identified by further complementation if necessary. The complementation groups and the rescued plasmids along with accession no. for the complementing ORFs are presented in Table 4.

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<td>p637</td>
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Table 4: *pyd1* mutants divided into their respective complementation groups and the complementing plasmids.  
Parental strains are $ = Y156, others = Y159
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</table>
| Bold = | MATa | Italic = MATα | (-) = same mating type | + = complementing mutants | - = non-complementing mutants.
DNA sequence analysis
Nucleotide sequence analysis and protein alignments were done with WinSeqEZ ver. 1.0 (F. G. Hansen unpublished) and ClustalX ver. 1.8 (Thompson et al., 1997). Database searches were performed using the default setup at the BLAST network services at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST/).

Gene disruptions
Replacement cassettes with very long flanking homology regions (approx. 500 bp) were used to disrupt the genes. The homology regions were designed so correct integration would result in removal of the start codon and at least 2/3 of the targeted ORF. The dominant cassette (kanMX3) from the plasmid pFA6-kanMX3, which confers geneticin (G418) resistance, was used (Wach et al., 1994). PCR amplification was performed with Pfu polymerase (Stratagene) from wild type genomic DNA with the oligos designed for amplification of two parts of the gene to be disrupted. All oligos used are presented in Table 1. For each gene two 500 bp DNA products corresponding to two (5’ and 3’) parts of the genes, were produced with 25-bp extensions (underlined in the oligos in Table 1) homologous to the kanMX3 cassette. A 1500 bp DNA product corresponding to the kanMX3 cassette was produced using pFA6-kanMX3 as template. In a second PCR amplification, the two 500 bp parts of the genes, where fused to the kanMX3 cassette using the outer primers. The resulting linear fragments of each 2500 bp were used to transform cells using electroporation as described in Gojkovic et al. (Gojkovic et al., 2000), and selected on G418 plates. Correct integration of these inserts was confirmed by PCR.

Growth conditions for HPLC experiments
Y159 was grown in uracil N-minimal media. All pyd mutants were grown in proline/uracil N-minimal media. Cells were harvested (1500 x g, 5 min) and washed with SD (-ammonium sulfate) media. Incubation with [2-14C] and [6-14C] labelled uracil was done in SD (-ammonium sulfate) media + 7 mM uracil (9:1), so final concentration of uracil was 700 µM. Incubation volume varied from 200 – 500 µl and the labelled uracil was added in 1:50 – 1:100 ratio either separately or both at the same time. It was found that > 1 x 10^8 cells / 250 µL was needed. If less was used,
none or very little radioactivity was detected in the cell fraction. Consequently, the nucleotide pools were almost undetectable on the HPLC. Incubation with labelled uracil was done for 30 – 60 min.

Sample preparation
Preparation was done at 4°C. After incubation, cells where harvested (14000 rpm, 1 min). 500 µL 10% trichloroacetic acid was added to the pellet, then it was vortexed and put on a spinning wheel for incubation (15-30 min, spinning). The tube was vortexed (10-15 s) and then spun down (14000 rpm, 1 min). The supernatant was transferred to a tube containing 700 µL Freon/trioctylamine (1:0.28), vortexed (30 s) and spun down (14000 rpm, 1 min). The top fraction was transferred to a tube containing 500 µL Freon/trioctylamine, vortexed (30 s) and spun down (14000 rpm, 1 min). The top fraction was transferred to a new tube. Samples were analyzed directly from fresh preparations.

HPLC conditions
Two columns were used. ZIC-HILIC (SeQuant, PEEK 150 x 4.6 mm, 200 Å, 5 µm), ZIC-pHILLIC (SeQuant, PEEK, 100 x 4.6 mm, 200 Å, 5 µm). Both columns were run isocratically with different buffers ranging between 80-90 % acetonitrile (ACN) and 2-10 mM ammonium acetate (AmAc) (pH~8) or ammonium carbonate pH 9.6. Detection was done with a UV spectrophotometer (set at 260 nm) and a continuous liquid scintillation counter. For GC/TOFMS analysis, samples were derivatized by trimethylsilylation before electron impact spectrunms were recorded.

RESULTS
Utilization of uracil as sole nitrogen source is independent of PYD2 and PYD3
S. kluyveri was mutagenized to obtain strains, which cannot degrade uracil. Screening of >50000 colonies yielded a total of 45 mutants failing to utilize uracil as sole nitrogen source. When a phenotypic test of the mutants was done on different media, all mutants could grow on DHU as sole nitrogen source. This was surprising, since pyd2 and pyd3 mutants were also expected in the mutant pool, and these should not grow on DHU as nitrogen source, if the degradation pathway was the same as in
mammals (FINK 1952). Two knock-out strains, Y986 \textit{Δpyd2} and Y1046 \textit{Δpyd3}, with directed gene disruption of the \textit{PYD2} and \textit{PYD3} loci, respectively, were constructed. When growth tests were made, a remarkable difference between the previously described \textit{pyd2} (Y1019) and \textit{pyd3} (Y1021) strains and the new \textit{Δpyd2} and \textit{Δpyd3} was observed (see Table 5). Both Y986 and Y1046 could grow on uracil as sole nitrogen source, while Y1019 and Y1021 could not. Apparently, the previously described \textit{pyd2} and \textit{pyd3} strains were double mutants (Gojkovic et al., 1998).

### Table 5: Growth of \textit{pyd2}, and \textit{pyd3} mutant strains on uracil (URA), dihydrouracil (DHU), beta-uridopropionate (BUP) and beta-alanine (BAL) media. +++ = good growth, - = no growth. Y1019 and Y1021 are apparently double mutants.

<table>
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<th>Mutant</th>
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<th>DHU</th>
<th>BUP</th>
<th>BAL</th>
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<td>+++</td>
<td>+++</td>
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<tr>
<td>Y1019 \textit{pyd2}*</td>
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<td>-</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Y1046 \textit{Δpyd3}</td>
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<td>-</td>
<td>-</td>
<td>+++</td>
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<tr>
<td>Y1021 \textit{pyd3}**</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
</tbody>
</table>

* (Gojkovic et al., 2000), ** (Gojkovic et al., 2001)

### Genetic loci involved in uracil utilization

The 45 \textit{pyd} mutants (Table 3) were analyzed by interallelic complementation tests. This test grouped them into six different complementation groups termed \textit{pyd11,12,13,14,15,16} with 9, 6, 14, 11, 4 and 1 number of mutants, respectively. Six plasmids were rescued from the genomic library, one from each complementation group. Each plasmid could complement one specific \textit{pyd} mutant, except for the plasmid (P637) complementing \textit{pyd13} mutants, which could also complement \textit{pyd15}. Apparently, \textit{PYD13} and \textit{PYD15} could belong to the same locus.

The plasmid inserts were sequenced and examined for the presence of ORFs. If the insert contained more than one ORF, the “right” one was elucidated by further complementation experiments. Mutant strains carrying gene disruptions in each of the six putative \textit{PYDIX} genes (elucidated on the basis of the insert analysis) were created (Table 2). The resulting \textit{ΔpydIX} strains all failed to utilize uracil as sole nitrogen source (Table 6) and therefore confirmed the previous results.
Chapter 4

**Table 6:** Growth of the pyd mutants on different nitrogen sources.

<table>
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**Pyd11p and Pyd14p are conserved proteins**

Plasmid P540 complements the \(\text{pyd}11\) mutants. It contains an ORF termed \(\text{PYD}11\) encoding a protein which contains a putative GTP cyclohydrolase II motif (see Appendix). Pyd11p has low homology (28 % identical) to GTP cyclohydrolase II (YBL033 Cp, RIB1p) from \(S.\ cerevisiae\), but high identity to a group of putative cyclohydrolases found in fungi and bacteria. The residues conserved in both Pyd11p and GTP cyclohydrolase II proteins belong to the active site of the cyclohydrolase.

Plasmid P722 complements the \(\text{pyd}14\) mutants. It contains an ORF termed \(\text{PYD}14\) encoding a protein, which has no assigned function and no conserved domains (see Appendix). A BLAST homology search yielded homologous proteins in fungi and bacteria, and interestingly \(\text{PYD}11\) and \(\text{PYD}14\) genes are always found together in the analyzed organisms.

**\(\text{PYD}13,15\) is homologous to the DUR1,2 gene from \(S.\ cerevisiae\)**

Plasmid P637 was found to complement both the \(\text{pyd}13\) and \(\text{pyd}15\) mutants. It contains a large ORF encoding a protein of 1830 amino acids (see Appendix). The protein has a high identity (74 % identical) to the \(S.\ cerevisiae\) bi-functional urea amidoloyase encoded by the \(DUR1,2\) gene. Plasmid P638 complements the \(\text{pyd}15\) mutants, but not the \(\text{pyd}13\). It contains an ORF encoding a protein of 1046 amino acid identical to a truncated version of Pyd13p (Figure 2). The genotype of both types of
mutants was confirmed by their inability to utilize urea or allantoin as sole nitrogen sources (Table 6). The Pyd13,15 protein consists of 5 domains, where the first domain is the allophanate hydrolase (Dur2 part) and the remaining four are responsible for binding of biotin and ATP and bicarbonate dependent urea carboxylase (Dur1 part). The ORF on P638 consists of the Dur2 part and the biotin motif from the Dur1 part.

**PYD12 encodes a putative Zn(2)Cys(6) type transcription factor/regulator**
Plasmid P471 complements the pyd12 mutants. It contains one ORF encoding a putative Zn(2)Cys(6) transcription factor (see Appendix). Its closest homolog in S. cerevisiae is the YDR520C gene product (52 % identical), but the S. kluyveri protein contains two putative introns (536-686 and 1561-1662) elucidated based on sequence homology. The S. cerevisiae gene has been connected to caffeine sensitivity (Akache et al., 2001), but otherwise does not have any known function.

**PYD16 is homolog to the FUR1 gene from S. cerevisiae**
Plasmid P731 complements the pyd16 mutant. It contains one ORF encoding a protein which is identical (87 %) with S. cerevisiae uracil phosphoribosyltransferase (see Appendix). This mutant is able to grow on uridine as sole nitrogen source, while the pyd11,12,13,14,15 cannot (Table 6). The ∆pyd16 strain on the other hand cannot use uridine as sole nitrogen source.

**Sequence analysis of homologues genes in other organisms**
Pyd11,12,14 proteins were checked against the reference protein database (refseq), and a number of hits were found. Accession numbers for the homologous sequences
are presented in Table 7. A total of 20 species (10 fungi and 10 bacteria) were identified as having both Pyd11p and Pyd14p, and none contained only one of them. Only four other species (all fungi) were found to contain Pyd12p, and two of those (S. cerevisiae and C. glabrata) does not have the PYD11 and PYD14 loci.

After identification of the 22 species having at least one of the PYD11, PYD12 or PYD14 genes, the corresponding genomes were analysed for the three last genes. In order to do so, the Pyd13,15 protein, because of its size and multidomain structure, was split into five domains (see Appendix), before doing the homology search. The AHS1 and AHS2 domains were used as positive indicators of Pyd13p, while the amidase domain was used for Pyd15p. Pyd16p (Fur1p) was found in all the 22 organisms and usually in more than one copy. The multisubunit protein Pyd13,15 was found in six of the 12 fungi, while two species have a protein that lacks the Pyd15 part and did not have any other homologous proteins for the Pyd15 part. One bacterial species has two proteins with homology to the Pyd13 and the Pyd15 part, respectively, and these two proteins are located next to each other on the chromosome. Of the remaining nine bacteria only two (Bradyrhizobium species) have homologous genes for the Pyd15 part. These two strains also have four proteins with homology to each of the four domains of Pyd13, and these four proteins are located next to each other on the chromosome.

**Gene organization in fungi and bacteria**

An analysis of the genomic location of the PYD1 genes was done. In nine of the ten bacteria found to contain Pyd11p and Pyd14p encoding genes (Cyanobacteria bacterium Yellowstone B-Prime was the exception), these genes were either located next to each other or as overlapping loci (indicating a polycistronic mRNA). In all nine cases a putative UPP gene identified as the bacterial homolog of the yeast FUR1 gene was found downstream of the PYD11 and PYD14 loci. B. bacteriovorus had a putative uridine kinase (UDK) loci overlapping the UPP gene, indicating coregulation of these genes. In eukaryotes it’s relatively rare to see genes in the same pathway
### Table 7: Accession no. for Pyd1X-like proteins. The table only contains reference sequences (refseq database), and only organisms containing at least one of either Pyd11p, Pyd12p or Pyd14p are presented. Note that some organisms contain duplicated genes. Key: - = no homologous proteins, (-) = low homology

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* The two *Bradyrhizobium* strains contain what looks like an operon composed of four individual cistrons encoding each of the domains from the *S. kluyveri* Pyd13,15p UCA part.
located next to each other, but in S. pombe the PYD11 and PYD14 genes were found close to each other. They were flanked on one side by a bacteria-like UPP gene and on the other side by three genes (a putative Zn(2)Cys(6) protein, a yeast FUR1 homologous gene and a yeast FUR4 homologous gene). This clustering of genes was also found in e.g. N. crassa and Y. lipolytica, where PYD11 and UPP or PYD14 and UPP were clustered, respectively. These gene organizations are presented in Figure 3.

**Figure 3:** Organization of genes homologous to SkPYD11, SkPYD14 and SkPYD16/FUR1 in various organisms. In bacteria and yeast, PYD11 and PYD14 are located either as closely spaced or overlapping loci. Next to them is a putative UPP (bacterial type uracil phosphoribosyltransferase) or PYD16/FUR1 (yeast type uracil phosphoribosyltransferase). A: B. japonicum (NC_004463 REGION: 7957090..7963265), B: B. bacteriovorus (NC_005363 REGION: Comp(1452015..1457248)), C: S. pombe (NC_003424 REGION: 1831000..1844000), D: N. crassa (NW_047266 REGION: Comp(68218..83644)), E: Y. lipolytica (CR382131 REGION: 2440600..2446600). In B. bacteriovorus (B) the flanking genes are identified as mmsA (methylamionic semialdehyde dehydrogenase) and udk (uridine kinase). In S. pombe (C), both a bacterial upp and a yeast pyd16/fur1 gene are located nearby. The pyd16/fur1 gene in S. pombe (C) is flanked by a fur4 (uracil transporter) homolog and a Zn(2)Cys(6) motif protein.

**URH1** gene product is not involved in uridine degradation

The finding that pyd16 could grow on uridine but not uracil as sole nitrogen source, suggested uridine to be one of the first intermediates in uracil degradation. The only
way to obtain uracil from uridine is through the enzyme uridine hydrolase (or uridine phosphorylase in higher eukaryotes). To test if uridine needed to be metabolized to uracil, a putative uridine hydrolase encoding gene (SkURH1) was disrupted. The ∆urh1 strains (Y1172-Y1175) could utilize uridine and uracil as sole nitrogen source. This strongly suggests that uracil is not the first substrate in the catabolism of uridine.

Results from HPLC experiments
Separation of the reference compounds uracil, BUP and BAL was achieved on the ZIC-HILIC column. When a running buffer consisting of 80 % ACN and 10 mM AmAc pH 8 was used at 1 mL/min, the retention times were 2.8, 8.0, and 17.2 min, respectively. DHU had the same retention time as uracil in all tested buffer/column systems.

The best separation of uracil, urea and uridine was found on the ZIC-pHILIC column using a running buffer consisting of 90 % ACN and 2 mM ammonium carbonate pH 9.6 at 1 mL/min. The retention times were 4.5, 6.3 and 6.8. The buffer was relatively unstable, and after 5-10 runs (3-5 hours), there was a shift in retention times and urea and uridine were coeluting.

Y159 and the four pydIX mutants, Y954 (pyd11), Y852 (pyd13), Y814 (pyd14) and Y960 (pyd15), were grown in uracil-containing media and incubated with uracil labeled at either 2-C or 6-C (see Materials and Methods). A total of seven different peaks were identified (designated A – G). Figure 4 shows an example of elution profiles from the pHILIC column of sample and media fractions. Compound A was identified as urea based on retention time, coelution with urea as internal standard, and supported by the fact that A was only labelled at 2-C and only found in the pyd13,15 mutants, which are unable to degrade urea (see Table 6). Samples containing A were subjected to GC-MS and urea was positively identified. B was the only compound showing UV absorbance at 260 nm, and was identified as uridine, based on coelution with uridine as internal standard.
Figure 4: Elution profiles of Y814 (pyd14) cellular (SAMPLE) and media (S.N.) fractions. The running and buffer conditions were: 1 mL/min, 90% ACN, 2 mM ammonium carbonate pH 9.6. The numbers (with the arrow) are in minutes. The peak eluting at approx. 4 minutes is uracil and it was usually not seen in the cellular fraction (though here it is present). The labelled peaks (B-G) are described in the text.
The buffer instability mentioned before made it difficult to identify peaks between samples, because they slowly changed position. Discrimination between compounds D and E was particularly difficult. Each strain had their unique pattern of compounds (see Table 8 and 9). Compounds A, B, D and E were found both in the cellular and in the media fractions, while C and F/G was strictly present in either the media or the cellular fraction, respectively. C, D and E were labelled only from the 6-C carbon. F was labelled at 2-C, but from the chosen experiments it was not possible to determine if it was also labelled at 6-C. The labelling of G could not be determined. The \textit{pyd11} mutant (Y954) only showed B, besides a small amount of C. This mutant may therefore have a defect in the early steps of the pathway. The \textit{pyd14} mutant on the other hand showed all compounds except A, and as \textit{pyd11} it showed B in the media fraction. It could be that Pyd14p is involved in the downstream steps.

It is important to stress, that uracil was usually not detected in the cells. Apparently, the imported uracil gets metabolised rapidly, and the intercellular pool of uracil is therefore primarily in the ribosylated state (uridine, UMP, UDP and UTP) which all could be detected in another separation system.
### Table 8: Presence of various compounds in the cell fraction. Identified compounds are A = urea, B = uridine. The rest are unidentified. (+) = small peak detected in the experiment.

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### Table 9: Presence of various compounds in the media fraction. Identified compounds are A = urea, B = uridine. The rest are unidentified. (+) = small peak detected in the experiment.

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DISCUSSION

A “novel” pathway

*S. kluyveri* can use uracil and all products of the reductive pyrimidine catabolic pathway as sole nitrogen sources (LaRue and Spencer, 1968). Previous studies on the *S. kluyveri* uracil degradation pathway resulted in the characterization of two genes (*PYD2* and *PYD3*), which encode DHP and BS, respectively (Gojkovic et al., 2000; Gojkovic et al., 2001). These data strongly suggested the presence of the reductive pyrimidine catabolic pathway in this organism. In order to identify and characterize the first enzyme encoded by *PYD1*, random mutagenesis followed by screening for mutants, which were unable to utilize uracil as sole nitrogen source, was performed. The resulting 45 mutants were analysed for growth defects on DHU, and surprisingly all were found to grow normally, indicating that none of the mutants were *pyd2* or *pyd3*. When the *PYD2* and *PYD3* loci were knocked-out, the resulting ∆*pyd2* and ∆*pyd3* strains were still able to grow on uracil as sole nitrogen source. This proves that *S. kluyveri* degrades DHU via the two enzymes, *PYD2* and *PYD3*, while uracil is degraded via the Pyd1 enzyme(s). This new pathway explains why no DHPDH activity could be measured in the cell free extracts (data not shown) and supports the previous observation that no DHPDH is not present in the *S. kluyveri* genome. It also explains why the Pyd2p activity is low in uracil-grown cells and high in DHU- and BUP-grown cells consistent with the observation that the *PYD2* mRNA is induced by DHU and to some degree BUP, but not by uracil (Gojkovic et al., 2000).

Five genes are involved in the “novel” pathway

Mutants strains unable to use uracil as sole nitrogen source belonged to six different loci. Two of loci (*PYD13* and *PYD15*) were found to be located within the same ORF, termed *PYD13,15*. A plasmid (P638) containing only the *PYD15* part of the *PYD13,15* gene could complement *pyd15*, but not *pyd13*. This proves that the two loci work independently, or at least that the Pyd15 part is active without the Pyd13 part. In strains with gene disruptions (∆*pyd1X*), the same uracil phenotype was observed as for *pyd1X* mutants confirming the identity of the genes.
**PYD11 and PYD14: Conserved elements found in fungi and bacteria**

Homologs of the two **PYD11** and **PYD14** loci, were found in 10 fungi and 10 bacterial species, and in nine of the ten bacteria they were located next to each other. The genetic structure with overlapping loci, indicated that the genes were part of a polycistron, which suggests coregulation at the transcription level. The fact that out of the six loci identified only **PYD11** and **PYD14** lack homologs in *S. cerevisiae*, suggests that these two loci are the core components and highly specific for this “new” uracil degradation pathway. The Pyd11 protein has a GTP cyclohydrolase II motif, with many of the functional residues conserved, while the Pyd14 protein sequence reveals no known structural/functional motifs. The striking resemblance of Pyd11p and GTP cyclohydrolase II indicates that the substrate may be a phosphoribosylated species.

**PYD16: Further indications of phosphoribosylated species as intermediate product**

**PYD16** was highly identical to *S. cerevisiae* **FUR1** gene. This was quite puzzling, since the background strain Y156 was ura3, and therefore totally dependent on **FUR1** salvage of pyrimidines. When assayed for UPRT activity, Y811 had the activity, but at 50-75 % of the level of Y156 (unpublished). The finding of this salvage enzyme as a part of the uracil degradation, indicates that uracil needs to be ribosylated, before a breakdown of the pyrimidine ring occurs, or that uracil transport in general is connected to the **FUR1** gene. In *S. cerevisiae*, it has been shown that the regulation of the uracil transporter (Fur4p) by uracil is independent of the **FUR1** locus (Seron et al., 1999). This was interpreted as normal transporter-mediated uracil entry into the Δ*fur1* cells, even though an actual uracil flux measurement was not done. It seems that the function of **PYD16/FUR1** would be to produce UMP. That uracil is transferred to UMP and not remaining at the nucleobase level (free uracil) is supported by **pyd16** and Δ*urhl* ability to utilize uridine as nitrogen source, and from the lack of any measurable uracil pool in cell fraction from wild type or **pyd1** mutants. The Δ*pyd16* strain could not utilize either uracil or uridine. This indicates an influence of uracil salvage on uridine salvage. A similar finding was observed when two recessive **FUR1** mutations (**fur1-1**, **fur1-6**) in *S. cerevisiae* were isolated (Jund and Lacroute, 1970).

Both mutants were 5-fluorouracil (5-FU) resistant, but the **fur1-1** mutant was as 5-
fluorouridine (5-Furi) resistant as a \textit{fui1} mutant (uridine transport mutant), while the \textit{fur1-6} mutant was sensitive as wild type cells. This indicates that the \textit{fur1-1} mutation abolished uridine salvage, while the \textit{fur1-6} mutation did not. The fact that \textit{S. cerevisiae \textit{ura3 \Delta fur1}} is still able to grow on rich media (Seron et al., 1999), indicates a functional cytidine salvage (through the action of cytidine deaminase and uridine kinase encoded by \textit{CDD1} and \textit{URK1}, respectively).

**PYD13 and PYD15: Urea as the terminal degradation product**

\textit{PYD13} and \textit{PYD15} were found to be highly identical to the \textit{S. cerevisiae DUR1,2} locus. Growth test on urea and allantoin confirmed this (Table 6). The involvement of urea degrading enzymes in uracil degradation could indicate an oxidative degradation pathway. Since none of the identified \textit{pydIX} loci share any homology to the only identified gene in the oxidative pathway (encoding barbiturase), and the fact that \textit{S. kluyveri} cannot utilize barbiturate as sole nitrogen source strongly indicates another reaction mechanism than oxidation of the 6-C atom of uracil. Evolution of the bifunctional enzyme urea amidolyase, can be seen by the finding that the two functional parts are separated in bacteria (but fused in eukaryotes). Apparently, evolution resulted in an enzyme (Pyd13,15/Dur1,2) from five different subunits as seen in for example \textit{B. japonicum}. Firstly a urea carboxylase (UCA) was fused from four of the subunits as seen in \textit{R. metallidurans}, and finally, fusion of the allophanate hydrolase (AH) with UCA, to generate the yeast type urea amidolyase (UAL). Why modern yeast have evolved a urea amidolyase instead of the normal urease is not known. Because the whole allantoin pathway is induced by allophanate, which can be made by UCA and not urease, that compound seems to be central and important for yeast.

**PYD12: Putative Zn(2)Cys(6) type transcription regulator**

A putative DNA binding motif is found in Pyd12p, suggesting that Pyd12p can function as a transcription factor. These types of transcription factors are known to be the key components of carbon and nitrogen metabolism in yeast. Some are involved in nitrogen catabolite repression (NCR) and others in induction of specific catabolic pathways. In \textit{S. cerevisiae}, the \textit{DAL} genes provide examples of inducible NCR genes. They are induced by either the end-product of the allantoin degradation pathway,
allophanate or by the non-metabolizable structural analog, oxalurate (Cooper and Lawther, 1973; Sumrada and Cooper, 1974). The induction is dependent on the specific gene regulator proteins Dal81 and Dal82, and the global activator Gln3p, while the NCR is controlled by the global repressor Dal80 protein (ter Schure et al., 2000). Pyd12p might have a similar function as Dal81p/Dal82p, upregulating the PYD1X genes transcription in an inducer-specific manner. Since the PYD12 gene is also present in S. cerevisiae, which does not contain the PYD11 and PYD14 genes, and cannot utilize uracil, it may have a common regulatory role (connected to nitrogen metabolism) in both S. kluyveri and S. cerevisiae. It could be that uracil acts as an inducer of its own salvage. A 5-FU resistant phenotype would be expected from this scenario, which could lead to gene isolation previously. Some inducer regulated genes are known to have a Gln3p induced basal transcription level even when inducer or the corresponding transcription factor are absent. This basal level of transcription might render pyd12 cells 5-FU sensitive, but could be insufficient for assimilation of uracil to support growth with uracil as sole nitrogen source.

**Intermediates in the novel uracil degradation pathway**

The finding of no or extremely low intracellular concentrations of uracil in all strains tested, and the accumulation of uridine even in mutant strains grown with proline as nitrogen source, strongly indicate that uracil as a free nucleobase is not the central intermediate. It is more likely that a ribosylated species (uridine, UMP, UDP or UTP), serves as a substrate for the first catabolic enzyme. At the same time it seems that uracil uptake and the following phosphoribosylation are tightly coupled to the degradation pathway. Besides urea and uridine five other radioactive compounds were found, and they were neither DHU, BUP or BAL.

**Proposed novel uracil degradation pathway**

Based on the six genetic loci identified and the HPLC experiments, the following hypothetical pathway is proposed (see Figure 5). The uracil uptake is immediately followed by and coupled to the phosphoribosylation into UMP by Pyd16p/Fur1p. The
first “real” catabolic enzyme is Pyd11p, which hydrolyses the uracil ring on one of the ribosylated uracil species (UriX), uridine, UMP, UDP or UTP (UriX). The Pyd11p reaction is a hydrolysis of the N-1 C-6 bond yielding ureidomalonic semialdehyde (UMSA)-ribose-X (UMSA-RX). Unspecific or enzymatic hydrolysis splits the N-glucosidic bond of UMSA-RX to UMSA and Rib-X. Pyd14p hydrolyze UMSA to urea and malonic semialdehyde (MSA). Urea gets degraded to ammonia and carbon dioxide in a two step reaction by the enzymes Pyd13p and Pyd15p. The further fate of MSA is not known, but conversion to BAL is a possibility. Other reactions could involve coupling to CoA (MSA dehydrogenase), oxidation (malonate dehydrogenase) or decarboxylating and reduction (MSA decarboxylase and alcohol dehydrogenase)
(MSA) (6-C labelled). The terminal enzyme is the Pyp13,15p complex converting urea into CO\(_2\) and ammonia via allophanate. Malonic semialdehyde would also be expected to be further metabolized, but none of the \(PYD1\) genes would be involved, since they are focused on the nitrogen containing compounds. This might involve a mitochondrial dehydrogenase similar to mammalian methylmalonic semialdehyde dehydrogenase converting MSA to acetyl-CoA and CO\(_2\) or a reverse aminotransferase reaction converting MSA to beta-alanine (BAL). Oxidation or decarboxylation could also be a possibility. However, it is possible that no further metabolism of MSA takes place if it can be actively excreted. Pyd12p is not directly involved in the reactions, but serves as a positive regulator of one or several \(PYD1X\) genes.

In conclusion, based on genetic and HPLC data, the flow of intermediates is proposed to be uracil -> UMP -> UriX -> UMSA-RX [F or G] -> UMSA [F or G] -> urea [A] + MSA and metabolites [C,D,E].

ACKNOWLEDGEMENTS

I would like to acknowledge the following people, who were actively participating in specific parts of the work presented in this chapter.

Ph.D. Zoran Gojkovic, ZGene A/S, Agern Alle, Hørsholm, Denmark, for the initial characterization of \(pydIX\).

Ph.D.-student Silvia Polakova, Faculty of Natural Sciences, Comenius University, Bratislava, for helping with the mutant screening and crossing.

Ph.D. Yuriy Pynyaha, Institute of Cell Biology, NAS, Ukraine, for helping with gene disruptions.

Ph.D. Olof Bjørnberg, Cell- and Organism Biology, Lund University, Sweden for helping with gene disruptions.

Assistant Prof. Anders Hofer, Department of Medical Biochemistry and Biophysics, Umeå University, Sweden, for letting me use his HPLC instruments and for helping with buffer/column optimization.

Prof. Thomas Moritz, Umeå Plant Science Center, Umeå University, Sweden, for running the GC/MS experiments.
REFERENCES


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### Saccharomyces kluyveri PYD11 gene

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#### Molecular weight: 49332.1


**Area:** 251 – 421

**CDD:** COG0807, RibA, GTP cyclohydrolase II

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**Number of amino acids:** 444

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**Saccharomyces kluyveri Pyd11p sequence**

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**Number of amino acids:** 444

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**Molecular weight:** 49332.1


**Area:** 251 – 421

**CDD:** COG0807, RibA, GTP cyclohydrolase II
Appendix

Saccharomyces kluyveri PYD12 gene

1. ATGGCCCTCGACAACAGGGAGACTTTTTTTTGACAACTTGAAGCCCACTACGCAGAGCATGTTTTCGACACTGCAGCAGCAGCAGCAGT
2. ACTCATTTGAGGACTTTTTTCAGGACTTTTTTGACAACTTGAAGCCCACTACGCAGAGCATGTTTTCGACACTGCAGCAGCAGCAGT
3. GCTTTGACGTTGCGGAAAGACAGCAAGTGTTGAACTTGTGCACCAAGATTTACTGGCACTTGAACACCATTGGCGAAAAGCTAAACGAGG
4. ACAAACAAGTACCTCCCGGTCGCTAGCTACACCTGGCGAGTACTGGCACTTTCTCCTGTTGCTGCTTTTTGCGTACGACTTTTACTACAG
5. CATGATATGCCGCTCCTTCCTGACCGAGTTTTTCG

Gene

1. AG
2. ACTCATTTGAGGACTTTTTTCAGGACTTTTTTGACAACTTGAAGCCCACTACGCAGAGCATGTTTTCGACACTGCAGCAGCAGCAGT

Appendix

91

Saccharomyces kluyveri PYD12 mRNA

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5. CATGATATGCCGCTCCTTCCTGACCGAGTTTTTCG

AG

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Chapter 4

protein domain annotations on the fly.


Area: 18 – 59
Saccharomyces kluyveri

5401 GTGCCTGCTAAAAAATCCGGTAAGGTTTTGAAAATCGTTCACAAGAATGGTGACATGGTCGATGCCGGTGGCATAGTGGCTGTCATTCAGTAG

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5001 CGACCAAGTTGAATTTTATCCAGTCAGTGAAGAGGAGCTGGACAGATTTACCGAGGATTGCGAAAATGGTAAATTCCCAGTTCAAGTGGAAGAAAGTGTT

4901 CACCAGGTGGTTATCAATTGGTTGGTAGAACCATTCCAATTTGGGACAAGTTGAAGTTGGGTTCTCACTCACAAGAACACCCTTGGTTGTTGACCCCATT

4701 TAAAGACGTGGAAAACATGTTGTACAGTGCTAGATTCTTGGTCTTAGGTTTAGGTGATGTATTTTTGGGTGCACCATGTGCTGTGCCTTTAGATCCCCGT

4601 CTTTGGAATGTGTTACTCGTTACCAAGAAACTATTCGTTCTAAGGCTCCATGGTTACCAAATAATGTTGATTTTGTTGCTGAAGTTAACGATATCACTCA

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601 DITKYGGFKK YIDFLKQEEA KVKKPFETVL IANRGEIAVR IIKTLKKLNI RSVAVYSDPD

541 GEETGSQIQL EVYSVPKENF GEFISMVPEP LGIGSVELES GEWVKSFICE EFGYTQKGTV

481 SPEDSVKLAV VGAHLKGLPL YWQLEKVNAT YLGSPKTSKN YKLYALPKTG PILKPGLRRV

421 RPDGLPQGVT LIGKKFTDFA LLELANRYFK VAFPQGSRTF GKFIDRQVTT KDDELRGPDI

301 LARCLYEGAW VAERYEATKD FFATNPPESS LDPTVTSIIK TATKYDAADS FRYEYQRQGI

181 VPAALNNLIG LKPTKGLFSC SGVVPACKSL DCVSVFAMNL SDAERCFKVM AKPDLENDEY

61 LQSKANKQQL PLYGVPIAVK DNIDSKGSPT TAACPAFEYN PSADSTVVAL LKDAGAIVIG

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62 IASS EMFKTAKVAT KILDSYDYKP CAFEVTSPGA

IASS EMFKTAKVAT KILDSYDYKP CAFEVTSPGA

102 PYD13,15p sequence

Pyd13,15p sequence

Saccharomyces kluyveri PYD13,15 gene

RRVKACDR CRRKICDRC KFPCCNC1KR GLECTYDSK

Saccharomyces kluyveri pyridoxal 5'-phosphate-dependent phosphatase thiolation}

Raw text: Saccharomyces kluyveri PYD13,15 gene

Pyd13,15p sequence

Saccharomyces kluyveri pyridoxal 5'-phosphate-dependent phosphatase thiolation
Chapter 4

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1621 GOGOMCNIV AMSDPOCGVL VGRTIFIPWQ KLGSHQQRH PMHIIFFIPDQ EVFVPYSBEL
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1801 VFAPKSMYLV KHVHNgMv DQGIVAVTQ

Underlined sequence corresponds to the ORF found on p638.

Number of amino acids: 1830 (1046)

Molecular weight: 201073.0


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121 PEKCGGSQSG SAGAASGGQIV FALGGTGAQ SGQFPAALNL LGKFLPTKFL ECGOSYFAC
181 KSLDCVSVFA WMNLSAEACF KMVAKCTLE DYESRPLSPN LQPKYPKNTV IAIFKEVFWY
241 GETENPKLY KAENEKLVQG ASIYTDIFPEF LLLALRCYLV GAVAEYKTE KROFDFNNP
301 ESSLIIPTVS IIKATKYOAA AGFPHYEYQR GQLQVQFDQT LKQDULCVPM CQFLAAPYEE
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181 TVQWQGKAYF GDGQYFEFSP VENAHVFEQ GMD.condalgai AZERDCSLQ RMNQRIEDST
241 PAPNLGMETR TMQGAASEL GSLLKYICAG TVEFYDDERK DEVFLEVNA RLOQKFTPFE
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241 GDEPIVTSSD GFSLGQFQVO AVVPFAELMK VQQVKPGDSI QFVPFISYQVA RQLKESQDAD

Area: 1437 – 1650

CDD: pfam02682, AHS1, Allophanate hydrolase subunit 1

1 QACDRYCLVLE YQGNQMOLDN AYRINQLNL VYRKHTGVCY EMSQCCVRSL IEYDSQYISQ
61 GALLTDVLV ESYIQFDKNM SIKSRKFRLE FADLSTKEL CVTVKQYETIR SIKAPFNYW
121 QPVAEYNDIT HKDVEWNLKS APFLVLGGLD VFLGAPCAVF LDPHRALFLGK KYFPSRTYTK
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1 RLEDRAFTEDC EKNSFXFQVVE ESYQDHXKNYL KWNINIESI TRFQMSQCGA KADEFARLIQ
61 VANQKLESL TNLKASVEEY PEDAEVYSE YGQSNKMKMV SAGQTVTGG GLVTTAAMK
121 EMVPFKAGK KVLIKQVHNG DMVDAGGIVA VI

94
**Saccharomyces kluyveri PYD14 gene**

Number of amino acids: 439

Molecular weight: 48647.0


Area: 1 - 439

CDD: No hits found!
Saccharomyces kluveri PYD16 gene

\[
\begin{align*}
\text{Saccharomyces kluveri} & \quad \text{Pyd16p sequence} \\
\end{align*}
\]

Number of amino acids: 216

Molecular weight: 24373.3


Area: 6 – 215

CDD: COG0035, Upp, Uracil phosphoribosyltransferase
Phylogenetic relationship among PYD16/FUR1 homologous genes found in PYD11, PYD12 and PYD14 containing organisms. S. cerevisiae Ura5,10 proteins were used as the outgroup. Clustering in two groups is seen. Top cluster contains yeast FUR1-like proteins, bottom cluster contains bacterial/fungi UPP-like proteins.
CHAPTER 5

S. KLUYVERI PYD4 GENE ENCODES A
BETA-ALANINE:ALPHA-KETOGLUTARATE
AMINOTRANSFERASE

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ABSTRACT

In humans, beta-alanine (BAL) and neurotransmitter gamma-aminobutyrate (GABA) are transaminated by a single aminotransferase (AT) enzyme, BAL-AT I/GABA-AT (EC 2.6.1.19). Apparently, yeast originally also had a single enzyme, but the corresponding gene was duplicated in the *Saccharomyces/Candida albicans* lineage. In *S. kluysteri*, *SkUGA1* encodes a homolog of *S. cerevisiae*-specific GABA-AT and *SkPYD4* encodes an enzyme involved in BAL and GABA transamination. *SkPYD4* and *SkUGA1* were sub-cloned, over-expressed, purified and characterized. It was found that, like in other aminotransferases, the cofactor pyridoxal 5’phosphate (PLP) is needed for (enzymatic) activity. SkPyd4p uses preferentially BAL as the amino group donor ($V_{\text{max}}/K_m = 0.78 \ U \ mg^{-1} \ mM^{-1}$), but can also use GABA ($V_{\text{max}}/K_m = 0.42 \ U \ mg^{-1} \ mM^{-1}$), while SkUga1p only uses GABA ($V_{\text{max}}/K_m = 4.01 \ U \ mg^{-1} \ mM^{-1}$). Like other GABA-ATs, SkPyd4p and SkUga1p use alpha-ketoglutarate, and not pyruvate as the amino group acceptor.

While mammals degrade BAL and GABA with only one enzyme, but in different tissues, a unicellular yeast has developed two different genes/enzymes, so that it can apparently distinguish between the two reactions in a single cell.
INTRODUCTION

In biological systems, beta-alanine (BAL) plays a major role as a precursor of pantothenic acid, as constituent of dipeptides like anserine and carnosine, and as an intermediate in the reductive degradation of pyrimidines. Because of its chemical similarity to the major inhibitory neurotransmitters, gamma-aminobutyrate (GABA) and glycine, and its presence in the brain, BAL is thought to have a similar function in animals. In mammals, uracil is degraded to BAL via three consecutive enzymatic steps (dihydropyrimidine dehydrogenase [DPD, EC 1.3.1.2], dihydropyrimidine amidohydrolase [DHP, EC 3.5.2.2] and beta-ureidopropionase [UP, EC 3.5.1.6]). In microorganisms, also other biochemical pathways provide BAL, e.g. Escherichia coli produces BAL by direct decarboxylation of aspartate, while Saccharomyces cerevisiae uses the break-down of polyamines (putrescine, spermidine and spermine) (Cronan, Jr., 1980; White et al., 2001; White et al., 2003).

Mammals degrade BAL to malonic semialdehyde (MSA) using either BAL:alpha-ketoglutarate (aKG) aminotransferase [BAL-AT I, EC 2.6.1.19] or D-3-aminoisobutyrate:pyruvate aminotransferase [BAL-AT II, D-BAIB-AT, EC 2.6.1.40]. Only BAL-AT I activity can be found in rat brain, and even though BAL-AT II activity is present in rat liver and kidney, BAL-AT I is the dominant enzyme also in these tissues (Kontani et al., 1999). The produced MSA is subsequently metabolized by methylmalonate semialdehyde dehydrogenase [EC 1.2.1.27] to acetyl-CoA (Goodwin et al., 1989; Tamaki et al., 1982; Ueno et al., 1990).

In humans, BAL-AT I and GABA-AT are identical (Schor et al., 2001). The gene is primarily expressed in brain, liver, pancreas and kidney, and the actual function in these tissues could be related to substrate availability eg. GABA-AT in brain (high GABA concentration) and BAL-AT I in liver (high BAL concentration). In rat, a processing protease in the liver modifies the enzyme in this tissue, leading to higher affinity for BAL, while leaving GABA affinity unchanged (Kontani et al., 1999; Ohyama et al., 2004).

Even though S. cerevisiae can synthetise BAL from polyamines, it cannot use it as sole nitrogen source (Di Carlo et al., 1952). GABA, on the other hand, is readily used as sole nitrogen source by the action of UGA1 and UGA2 encoding GABA-AT and
succinic semialdehyde dehydrogenase, respectively (Ramos et al., 1985). Transport of GABA across the cell membrane is facilitated by the UGA4 gene product (Vissers et al., 1989). The whole GABA catabolic pathway is regulated by the transcriptional activator UGA3 (Andre, 1990). Many yeast strains, like Saccharomyces kluyveri and Schizosaccharomyces pombe can utilize BAL as sole nitrogen source, while S. cerevisiae cannot (LaRue and Spencer, 1968). S. kluyveri has recently become a useful model to study the synthesis of BAL (Gojkovic et al., 1998; Lundgren et al., 2003), and now this yeast is employed as a model for BAL degradation. A genetic and enzymatic characterization of a BAL-AT (encoded by PYD4) and a GABA-AT (encoded by UGA1) from S. kluyveri is presented. These two enzymes are compared with GABA-AT from S. pombe and S. cerevisiae. These are the first genetic and enzymatic data presented on BAL degradation in fungi.

MATERIALS & METHODS

Materials
Uracil (U0570), dihydrouracil (D7628), beta-ureidopropionate (C3750), beta-alanine (146064) and gamma-aminobutyrate (A5835) were purchased from Sigma. Yeast nitrogen base w/o amino acids and ammonium sulfate were obtained from Difco. Oligos were purchased from DNA Technology, Denmark. Sequencing was done by MWG Biotech, Germany.

Strains and growth media
The yeast and bacterial strains used in this study are presented in Table 1. The strains were grown in YPD medium (1 % yeast extract, 2 % Bactopeptone, 2 % glucose) or synthetic defined (SD) medium (1 % succinic acid, 0.6 % sodium hydroxide, 2 % glucose, 0.67 % yeast nitrogen base w/o amino acids and ammonium sulfate) supplemented with different nitrogen sources (0.5 % ammonium sulfate, 0.1 % for all other types). For solid medium (plates) 2 % agar was added. All percentages are w/v. The Escherichia coli strain XL1-Blue (Stratagene) was used for plasmid rescue, TOP10 was used for cloning and BL21 Star™ (DE3) was used for overexpression of fusion proteins, respectively. All bacteria were grown at 37°C in Luria-Bertani medium supplemented with ampicillin (100 mg/liter) for selection (Sambrook et al.,
G418 selection media consisted of YPD supplemented with 75 mg/L of G418 (SIGMA G5013).

**Mutagenesis**

Yeast mutants were generated from the strains Y156 and Y159 with ethyl methanesulfonate (EMS) as described in Gojkovic et al. (Gojkovic et al., 1998). Mutagenized cells were plated on YPD plates (100-200 colonies per plate) and grown for 2-3 days at 25°C. The plates were replicated onto new plates containing ammonium sulfate (control) or uracil (DHU) as sole nitrogen source. After 5-7 days at 25°C colonies were selected based on their inability to grow on DHU plates compared to the control plates. Putative mutants were tested for growth on BUP and BAL as sole nitrogen source.

**Complementation of Y947 with genomic library**

The used *S. kluyveri* wild type genomic library was prepared by F. Lacroute and based on the shuttle vector pFL44S (Bonneaud et al., 1991). Y947 was transformed with the library DNA using the electroporation procedure (Gojkovic et al., 2000) and plated on media containing beta-alanine as sole nitrogen source for selection. A number of transformants were tested for plasmid loss, before rescue of the plasmid, P733, into *E. coli* strain XL1-Blue. Sequencing was done using the primers M13rev-29 and M13uni-21.

**DNA sequence analysis**

Nucleotide sequence analysis and protein alignments were done with WinSeqEZ ver. 1.0 [F. G. Hansen unpublished] and ClustalX (Thompson et al., 1997). Database searches were performed using the BLAST network services of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).
## Table 1: Strains description

### Saccharomyces kluweri strains

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<th>Designation</th>
<th>Reference/origin</th>
<th>Genotype</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Diploid, prototroph</td>
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</tr>
<tr>
<td>Y090</td>
<td>L. Marsch, MYA-2152</td>
<td>MATα thr</td>
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<td>MATα ura3</td>
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<td>haploid, aux ura1 ::KanMX3 pyd4 ::KanMX3</td>
<td>Random spore</td>
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### Escherichia coli strains

<table>
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<th>Reference/origin</th>
<th>Genotype</th>
<th>Comment</th>
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</thead>
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<td>XL-1 Blue</td>
<td>STRATAGENE</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’ proAB lacIqZΔM15 Tn10 (TetR)]</td>
<td></td>
</tr>
<tr>
<td>TOP10</td>
<td>INVITROGEN</td>
<td>F mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG</td>
<td></td>
</tr>
<tr>
<td>BL21 Star™(DE3)</td>
<td>INVITROGEN</td>
<td>F ompT hsdS (rB mB ) gal dcm rne131 (DE3)</td>
<td></td>
</tr>
</tbody>
</table>
Disruption of the PYD4 and UGA1 genes.

Replacement cassettes with very long flanking homology regions (approx. 500 bp) within the ORF were used to disrupt the two genes. Upon correct integration approx. 1/3 of the ORF (the middle part) would be exchanged with the resistance cassette. The dominant cassette KanMX3, which confers geneticin resistance was used. PCR amplification was performed with Pfu polymerase (Stratagene) from wild-type genomic DNA (Y057) with the oligonucleotides:

PYD4 1-5' (CCTCTTATTCTGCTGCTGAACTT)
PYD4 1-3' (GACCCGGCGGGGACGAGGCAAGCTTCAGTGCTAAAGACCTACCATTTC)
PYD4 2-5' (CGAGCCCTGCCACGGCTCTGCGCCCCATGAACACTGGAACCTAAGTAACTC)
PYD4 2-3' (CTAACGGTAAAGTTGTCTCAATGTGTT)
UGA1 1-5' (ATGACTGTTTGCGAAAGCTACTACC)
UGA1 1-3' (GACCCGGCGGGGACGAGGCAAGCTTTTCATTTTCTTCAGCGAAAGCTCAG)
UGA1 2-5' (CGAGCCCTGCCACGGCTCTGCGCCCCATACATTGATGAAAGTFTCAAAACTGG)
UGA1 2-3' (TTCAAATGTCAACAAAGATGTGCAAGCTTGCCTCGTCCCCGCCGGGTC)
KanMX3 5' (GGGCGCAGAGCCGTGGCAGGGCTCG)
KanMX3 3' (AAGCTTGCCTCGTCCCCGCCGGGTC)

and on pFA6-KanMX3 plasmid with the oligonucleotides

KanMX3 5' (AAGCTTGCTCTGCTCCCGCAGCGGTC),
KanMX3 3' (GGGCAGAGCCGTGGCAGGGCTCG)

generating four 500 bp DNA products corresponding to two parts of the genes, with 25-bp extensions (underlined) homologous to the KanMX3 marker, and one 1500 bp DNA product corresponding to the KanMX3 cassette. In a second PCR amplification, the two 500 bp parts of the genes, were fused to the KanMX3 cassette using the outer primers. The two resulting linear fragments of each 2500 bp was used to transform S. kluyveri cells using electroporation as described in Gojkovic et al., 2000, and selected on G-418 plates. Correct integration was confirmed by PCR.

Cloning, expression and purification procedures

Proteins were expressed using the pET151/D-TOPO expression system (Invitrogen K151-01). The expression vector pET151 adds a 33 amino acid N-terminal his-tag (MHHHHHHHGKPIPNPLLGLDSTENLYFQGIDPFT) to the expressed protein. Total DNA from S. kluyveri (NRRL Y-12651) and S. cerevisiae (NRRL 12632) and a commercial cDNA library from S. pombe were used as template. The cloning was done as described by the manufacturer, and clones were checked for correct insertion by PCR and restriction analysis, before sending for sequencing. The following
oligonucleotides for SkPYD4 (DQ512721), SkUGA1 (DQ512722), ScUGA1 (NP_011533) and SpUGA1 (NP_594905) were used:

SkPYD4-5’ CACCATGCCCTCTTATTCTG
SkPYD4-3’ CTAACCGTAAAGTTGTCTCAATG
SkUGA1-5’ CACCATGACTGTTTGCAAG
SkUGA1-3’ CTAACGGAGAAACACCTTTTC
ScUGA1-5’ CACCATGTCTATTTGTGAACATAC
ScUGA1-3’ TCATAACATAACTGATTGCC
SpUGA1-5’ CACCATGTCTTCTACTGCCAC
SpUGA1-3’ TTAATCAATCGTCAATTTTCTTGAAC

The resulting plasmids were termed pET151-SkPYD4 (P895), pET151-SkUGA1 (P896), pET151-ScUGA1 (P897) and pET151-SpUGA1 (P898).

**Purification procedure (Pyd4 and Uga1 proteins)**

Expression: BL-21(DE3) were transformed as described by the manufacturer. A preculture was made by inoculation of the transformation in 10 mL LB media [100 µg/mL ampicillin, 100 µM PLP] and overnight growth (37°C) with shaking. The preculture was transferred to 1 L LB media [100 µg/mL ampicillin, 100 µM PLP] and the culture was grown at 37°C with shaking till the OD₆₀₀ was 0.5-0.6. The temperature was lowered to 25°C, IPTG was added to a final concentration of 100 µM, and the culture was grown over night (25°C). Harvesting was done by centrifugation at 4,225 x g for 10 min, and the harvested cells were either stored at -80°C or processed directly. All procedures past the harvesting were done in the cold (ice or 4°C).

Cell disruption: The pellet was resuspended in 20 mL lysis buffer [50 mM potassium phosphate pH 7.0, 300 mM potassium chloride, 100 µM PLP, 1 mM dithiothreitol (DTT), Complete Minus EDTA Protease Inhibitor cocktail tablets (ROCHE Diagnostics)] and disrupted in a French Press (4 x 1,000 psi). Cell debris was removed by centrifugation (24,500 x g, 30 min).

Streptomycin treatment: Streptomycin sulfate (10 %) was added dropwise to a final concentration of 2 % with stirring. The precipitate was removed by centrifugation (24,500 x g, 30 min).
Desalting: The solution was desalted on a G-25 column. The column was equilibrated and run with buffer A [50 mM potassium phosphate pH 7.0, 300 mM potassium chloride, 100 µM PLP].

Affinity chromatography: The desalted solution was applied to a 4.5 mL Ni\(^{2+}\)-NTA (Chelating Sepharose Fast Flow from Amersham Biosciences). The column was equilibrated and washed with 200 mL buffer I [50 mM potassium phosphate pH 7.0, 300 mM potassium chloride, 100 µM PLP, 20 mM imidazole]. The protein was eluted by stripping the column with buffer B [50 mM potassium phosphate pH 7.0, 300 mM potassium chloride, 100 µM PLP, 10 mM EDTA].

(NH\(_4\))\(_2\)SO\(_4\) precipitation: The protein was concentrated by ammonium sulfate precipitation (and removal of Ni\(^{2+}\) and EDTA would also be achieved). Finely ground solid ammonium sulfate was slowly added to obtain 70 % saturation (0.472 g (NH\(_4\))\(_2\)SO\(_4\)/ml protein solution). The solution was stirred for 15 min prior to centrifugation (24,500 x g, 30 min). The precipitated protein was dissolved in 6 mL buffer S [100 mM potassium phosphate pH 7.0, 100 µM PLP].

Gel filtration: A final gel-filtration step was used to remove ammonium sulfate. The S-12 gel-filtration column was equilibrated and run with buffer S. Active fractions were pooled. Glycerol was added to a final concentration of 10 % (v/v), and protein was stored in 200 µL and 2 mL fractions at -80°C.

**Purification procedure (Malonic semialdehyde decarboxylase, MSADC)**

Expression, cell disruption, streptomycin treatment and desalting (G-25 column) is the same as above except all potassium was exchanged for sodium and no PLP was added.

Affinity chromatography: The desalted solution was added slowly to a slurry of Ni\(^{2+}\)-NTA material (Chelating Sepharose Fast Flow from Amersham Biosciences) on ice. The content was slowly swirled around for approx. 5 min, before carefully removing buffer by vacuum filtration, without letting the Ni\(^{2+}\)-NTA material run dry. Wash buffer [50 mM sodium phosphate pH 7.0, 300 mM sodium chloride, 10 % glycerol, 25 mM imidazole] was added, and the mixture was swirled around and vacuum filtered. The washing procedure was repeated several times. The column material was scraped of the filter and mixed with 5 mL of elution buffer [50 mM sodium phosphate pH 7.0, 300 mM sodium chloride, 10 % glycerol, 500 mM imidazole, 20 mM EDTA].
This mixture was then transferred to a 10 mL single use syringe, with a filter placed at the bottom. The mixture was placed at room temperature for half an hour with regular inversions of the syringe, before filtering the solution through the syringe. The solution was dispensed in a dialysis tube (10 kDa cut-off), covered with absorbant gel matrix, and incubated under thin-foil at 4°C to concentrate the protein solution. The volume was reduced to 2 mL before running a final gel-filtration on a S-12 column. The S-12 gel-filtration column was equilibrated and run with storage buffer [100 mM sodium phosphate pH 7.0, 10 % glycerol]. Protein fractions were pooled and stored in 200 µL and 2 mL fractions at -80°C.

Protein concentrations were determined based on the calculated extinction coefficient at 280 nm (www.expasy.org/tools/protparam.html) or by the colorimetric method of Bradford using bovine serum albumin as standard (Bradford, 1976). Protein purity and molecular mass determination was done by SDS-PAGE (Laemmli, 1970). Native protein mass was determined by running native gel electrophoresis at four different acrylamide concentrations (4, 6, 8 and 10 %). Gels were stained with Coomassie Blue [40 % ethanol, 1 % acetic acid, 1 g/L R-250 Coomassie Blue] and destained with 1 % acetic acid. Beta-amylase (200 kDa), bovine serum albumin, dimer (132 kDa), bovine serum albumin, monomer (66 kDa), and carbonic anhydrase (29 kDa) were used as protein standards.

**Spectroscopic measurements:**

All measurements were done on a Varian Cary3 UV/Visible spectrophotometer with Varian data analysis tools. The spectrometer was equipped with a temperature controled automatic multi-cell changer.

**Enzyme assays**

**Glutamate dehydrogenase stopped assay with quenching (GDH assay)**

The enzymatic activity of BAL-AT I type enzymes was determined in a discontinued assay system based on a previously described method (Weber et al., 1992). The first reaction (1) is catalyzed by BAL-AT I type enzymes, which converts BAL/GABA and aKG to malonic semialdehyde (MSA) or succinic semialdehyde (SSA) and glutamate respectively. The second reaction (2) is catalyzed by glutamate dehydrogenase (SIGMA G2626), which stoichiometrically converts glutamate to aKG and ammonia while reducing NAD\(^+\) to NADH. In the second reaction, aKG
originating from the first reaction is quenched by hydrazine, allowing the conversion of glutamate to go to completion.

The two reactions are:

\[ \text{BAL/GABA + aKG} \rightleftharpoons \text{MSA/SSA + glutamate} \quad (1) \]

\[ \text{glutamate + NAD}^+ + \text{H}_2\text{O} \rightleftharpoons \text{aKG + NADH + NH}_4^+ \quad (2) \]

The standard reaction mixture consisted of 100 mM potassium phosphate (pH 8.0), 100 µM pyridoxal 5'-phosphate (PLP, SIGMA P9255), aKG, BAL/GABA and 0.3 – 3 µg PYD4/UGA1 enzyme. The reaction was carried out in a total volume of 200 µL at 30°C for 5 min. The reaction was terminated by addition of 40 µL ice-cold stop mix (20 % TCA/0.6 M hydrazine) and placed on ice. After incubation on ice for 10 min, the reaction was neutralized with 20 µL 2 M potassium hydroxide. The second reaction mixture contained 260 µL first reaction, 640 µL 0.5 M glycine buffer (pH 9.0), 10 µL 0.5 mM ADP (SIGMA A5285), 80 µL 2 mM NAD\(^+\) (Boehringer Mannheim) and 10 µL glutamate dehydrogenase to a final volume of 1 mL. A measurement of the initial absorbance at 340 nm was taken before adding NAD\(^+\). The reaction was carried out until no further rise in absorbance at 340 nm was observed (approx. 1 hour). The amount of glutamate was calculated from the total change in absorbance at 340 nm using a molar extinction coefficient of NADH of 6.22 mM\(^{-1}\)cm\(^{-1}\). One unit (U) of BAL/GABA-AT was defined as the amount of enzyme which catalyzed the formation of 1 µmol of glutamate in 1 min.

**Succinic semialdehyde dehydrogenase coupled assay (SSADH assay)**

In a continuous assay, the succinic semialdehyde produced by GABA-AT (3) is converted by the NADP\(^+\)-dependent SSADH (4), a *E. coli* gabD gene product, to NADPH and succinate. The absorbance change at 340 nm is monitored. The following enzymatic reactions are involved:

\[ \text{aKG + GABA} \rightleftharpoons \text{glutamate + SSA} \quad (3) \]

\[ \text{SSA + NADP}^+ + \text{H}_2\text{O} \rightleftharpoons \text{succinate + NADPH} \quad (4) \]

The *gabD* gene cloned in the overexpression plasmid pET23a(+) (P899) was kindly provided by M.D. Toney (University of California, Davis), and the purification was
executed as described previously (Liu et al., 2004). The activity of the purified SSADH was determined at 30°C in a reaction mixture containing 10 µL 1/10 diluted SSADH, 10 µL 30 mM SSA (SIGMA S1505), 10 µL 20 mM NADP⁺ (SIGMA N0505), 970 µL 100 mM potassium phosphate (pH 7.0). One unit (U) of SSADH was defined as the amount of enzyme which catalyzed the formation of 1 µmol of succinate in 1 min. The SSADH activity was calculated to be 0.15 U µL⁻¹ based on the change in absorbance at 340 nm and a molar extinction coefficient of NADPH of 6.22 mM⁻¹cm⁻¹. The amount of SSADH in the assay was optimized, so SSADH was not limiting the reaction. One unit (U) of GABA-AT was defined as the amount of enzyme which catalyzed the formation of 1 µmol of SSA in 1 min. Standard GABA-AT assay conditions were 100 mM potassium phosphate (pH 8.0), 100 µM PLP, 0.3 units/mL SSADH, 0.4 mM NADP⁺, aKG, GABA and 0.5 – 9 µg SkPYD4/SkUGA1 enzyme. The assay could not be used to monitor the production of MSA from BAL-AT reaction.

Malonic semialdehyde decarboxylase coupled assay (MSADC assay)

A continuous assay coupling BAL-AT produced malonic semialdehyde (MSA) (5) with the reaction of Pseudomonas pavonacea 170 MSADC (6) was developed. The MSADC-generated acetaldehyde (7) was monitored by following the depletion of NADH at 340 nm using a NADH-dependent alcohol dehydrogenase (ADH) (Poelarends et al., 2003). The three coupled reactions are:

\[
\text{aKG + BAL} \leftrightarrow \text{glutamate + MSA} \tag{5}
\]

\[
\text{MSA} \leftrightarrow \text{acetaldehyde + CO}_2 \tag{6}
\]

\[
\text{acetaldehyde + NADH} \leftrightarrow \text{ethanol + NAD}^+ \tag{7}
\]

The overexpression vector pET(130) containing the P. pavonacea 170 orf130 encoding MSADC was kindly provided by C.P. Whitman (University of Texas at Austin) (Poelarends et al., 2003). The gene was cloned into pET101/D-TOPO (Invitrogen), using the 5’ primer CACCATGCCACTTCTCAAG and the 3’ primer GACGAGGTCCCCAGTC. The resulting plasmid (P900) was sequenced. Transformation, expression and purification was done as described above. Amounts of MSADC and ADH were optimized to concentrations which are not limiting for the
reaction. One unit (U) of BAL-AT was defined as the amount of enzyme which catalyze the formation of 1 µmol MSA per min. Since the BAL-AT catalyzes the limiting reaction, the activity can be calculated from the amount of NADH reduced to NAD\(^+\) using the extinction coefficient of NADH of 6.22 mM\(^{-1}\)cm\(^{-1}\). The standard BAL-AT assay conditions (1 mL total volume) were 100 mM potassium phosphate (pH 9.0), 0.2 mM NADH, 5 µL ADH (SIGMA A3263), 50 µL MSADC, aKG, BAL, 2 – 30 µg SkPyd4p/SkUga1p.

**Data analysis**

All data analysis was done using the commercial curve-fitting EnzFitter version 2.0 from Biosoft.

**RESULTS**

**Identification of PYD4 gene encoding a beta-alanine aminotransferase**

While screening EMS mutagenized cells on dihydrouracil plates, a mutant, Y947 (MATa ura3 pyd4), which could use neither dihydrouracil (DHU), beta-ureidopropionate (BUP) nor beta-alanine (BAL), but uracil as sole nitrogen source, was isolated. This mutant would normally be considered a double mutant, since both BUP and BAL can serve as a direct nitrogen source via the action of UP or BAL-AT. However, the complementation with the genomic library yielded plasmid P733, which could grow on all three nitrogen sources (DHU, BUP and BAL). An ORF, termed PYD4 (DQ512721), encoding a protein with 55 % identity at the protein level to *S. cerevisiae* UGA1 gene product, GABA-AT, was identified. A BLAST search of *ScUGA1* in the *S. kluyveri* genome (Cliften et al., 2003) revealed another homologous gene, termed SkUGA1 (DQ512722). Its gene product has 80 % identity to ScUga1p, and 57 % to SkPyd4p. Knock-out strains of both, *PYD4* and *UGA1*, were constructed in different background strains (Table 1). Two double mutants (Y1154 and Y1155) were made from crossing and sporulation of Y1042 (*Δuga1*) and Y1048 (*Δpyd4*) (Table 1). Strains were tested for their ability to utilize DHU, BUP, BAL and GABA as nitrogen sources (Table 2). The Y1048 (*Δpyd4*) strain showed low growth on BAL. However, in contrast to Y947 (*pyd4*) it could grow on both DHU and BUP. Y1042 (*Δuga1*) showed decreased growth on GABA, but was able to grow on the other
nitrogen sources tested. The double mutants Y1154 and Y1155 showed decreased growth on both BAL and GABA as sole nitrogen source. These results suggest that SkPYD4 is primarily involved in BAL degradation, but also has some influence on GABA degradation. SkUGA1, on the other hand, is only involved in GABA degradation. The remaining poor growth on BAL and GABA, when both genes are disrupted, indicates the presence of some unspecific background GABA-AT and BAL-AT activity in the cells.

Table 2: Growth of pyd4 and uga1 mutant strains on uracil (URA), dihydrouracil (DHU), beta-ureidopropionate (BUP), beta-alanine (BAL) and gamma-aminobutyrate (GABA) media. +++ = good growth, + = growth, +/- = some growth, - = no growth

<table>
<thead>
<tr>
<th>Mutant</th>
<th>URA</th>
<th>DHU</th>
<th>BUP</th>
<th>BAL</th>
<th>GABA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-947 (pyd4)</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Y-1042 (∆uga1)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+/-</td>
<td>+++</td>
</tr>
<tr>
<td>Y-1048 (∆pyd4)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Y-1154 (∆uga1 ∆pyd4)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+/-</td>
<td>+/-</td>
</tr>
</tbody>
</table>

Sequence analysis

Phylogenetic analysis of homologous proteins from yeast shows that the Pyd4p protein is only found in two other yeasts (Debaryomyces hansenii and Candida albicans). Both yeasts also contain a putative Uga1p encoding gene (Figure 1). When a search for Pyd2p and Pyd3p encoding genes was performed, it was found that S. kluiveryri is the only yeast having all three genes, C. albicans and D. hansenii lack PYD2, and K. lactis has PYD2 and PYD3, but lacks PYD4 (Table 3). A phylogenetic tree of all Pyd4/Uga1 homologous proteins found in fungi with mammalian BAL-AT I and BAL-AT II (DBAIB-AT) is shown in Figure 2. ScCar2p and ScArg8p are included to illustrate general phylogenetic relationships.
S. kluyveri PYD4 gene

Figure 1: Phylogenetic tree of yeast Pyd4p and Uga1p. Sk = S. kluyveri, Dh = D. hansenii, Ca = C. albicans, Yl = Y. lipolytica, Cg = C. glabrata, Eg = E. gossypii, Kl = K. lactis, Sc = S. cerevisiae, Sp = S. pombe. SpUga1p (NP_594905) was used as an outgroup.

Table 3: Homologous proteins in K. lactis, D. hansenii and C. albicans. Numbers in brackets show percent identity to the S. kluyveri protein.

<table>
<thead>
<tr>
<th>Specie</th>
<th>PYD2</th>
<th>PYD3</th>
<th>PYD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>K. lactis</td>
<td>XP_453052  (69 %)</td>
<td>XP_453223  (77 %)</td>
<td>No homolog</td>
</tr>
<tr>
<td>C. albicans</td>
<td>No homolog</td>
<td>XP_714639  (54 %)</td>
<td>XP_721100 (64 %)</td>
</tr>
<tr>
<td>D. hansenii*</td>
<td>No homolog</td>
<td>XP_458518  (53 %)</td>
<td>XP_460765 (61 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XP_460391  (53 %)</td>
<td></td>
</tr>
</tbody>
</table>

*There were two PYD3 homologous genes in D. hansenii (60% identical to each other).
Cloning and expression

The PYD4/UGA1 genes from S. kluuyveri, S. cerevisiae and S. pombe, were sub-cloned. Four expression plasmids (pET151-SkPYD4, pET151-SkUGA1, pET151-ScUGA1 and pET151-SpUGA1, respectively) were constructed using the pET151/D-TOPO cloning system. Sequencing confirmed correct cloning with no mutations, except for ScUGA1. Here a change (719 A->G) was found resulting in an amino acid change (H240R), when compared to the reference sequence NP_011533. In the original published sequence, position 719 is a guanine not adenine (Andre and Jauniaux, 1990). This is also noted in the SwissProt entry P17649 (even though the actual protein sequence in this entry has a histidine at position 240). The ScUGA1 sequence was deposited in genbank (DQ512723). All the other yeast Uga1 proteins used in the phylogenetic analysis including S. pombe have an arginine at that position.
so it is most likely that the reference sequence is wrong, and the pET151-ScUGA1 clone contains the wild-type sequence.

A typical purification of SkPyd4p from 1 liter culture yields approx. 20 mg pure protein. The SDS-PAGE gel in Figure 3 shows the different fractions during the purification. The purified protein fractions show only minor impurities (Figure 4). The molecular weight of the protein was determined to be 56-57 kDa based on SDS-PAGE gel, matching the calculated values of between 56.3-56.9 kDa. The purified proteins were dimers as determined by native gel. Some of the ScUga1 and SkUga1 proteins were seen as tetramers.

Absorption spectrum

The spectrum of purified SkPyd4p showed two maxima (340 nm and 410 nm) besides the 280 nm absorbance. When the pH varied between 6.6 and 8.6, a pH dependent shift of these maxima was observed (Figure 5). When the protein was dialysed against 3 times 1 L buffer S without PLP, the 340/410 nm absorption maxima disappeared (Figure 6). The protein without the 340/410 nm absorption was inactive.
When PLP was added to the dialysed protein the 340/410 nm absorption reappeared and the protein becomes active again. When the reconstituted protein is compared to buffer containing PLP in the same concentration (10 \( \mu \text{M} \)), there is a shift in the maxima from 330/390 nm for PLP to 340/410 nm in the reconstituted SkPyd4p.
indicating the formation of the Schiff base of the formyl group of PLP with the ε-amino group of a special lysine residue of the protein. From sequence alignment with *E. coli* GABA-AT it can be deduced that the special lysine of SkPyd4p forming the Schiff base must be K329.

**Enzyme kinetic measurements**

Three assays were used in the kinetic measurements. The GDH assay has the advantage that it is based on the determination of the amino acceptor product, i.e. both BAL and GABA can be measured. But it has the disadvantage that hydrazine has to be added to remove aKG from the first reaction, and this influences the glutamate detection. The detection efficiency for glutamate was tested at different aKG concentrations, and it was found that for aKG concentrations higher than 5 mM the detected glutamate decreased. This is not a problem when determining relative activities at a fixed aKG concentration. For a full kinetic analysis a broad spectrum of concentrations is needed, and therefore the varying sensitivity of the GDH assay has disadvantages and it is very time-consuming. The lack of sensitivity could be overcome to some degree by adding GDH enzyme in amounts, which were not practical for large series of kinetic analysis experiments. The SSADH assay, on the other hand, is a very sensitive, continuous assay with no interference between first and second reaction.

**pH optimum and substrate specificity**

A series of measurements at different pH was done on SkPyd4p (Figure 7). The pH optimum was close to 8.0 with approximately 70% activity at pH 7 and 9. This pH is in agreement with other GABA/BAL-AT, and was used for all assays. The enzyme’s stability at 4°C was tested and after 12 days 57% activity was left.

The purified enzymes were tested for their ability to use either BAL or GABA as amino donor (Table 4). There is a little inconsistency in the relative activities determined using the different methods, but in general it can be seen that SkPYD4 is more active with BAL, while the other three enzymes show only GABA-AT activity. In addition, SkPyd4p does not use pyruvate as amino acceptor.
A full kinetic analysis on SkPyd4p and SkUga1p was done. Since SkUga1p had so little activity with BAL, only SkPyd4p was analyzed with BAL. A lot of effort was put into analysis using the GDH assay. At aKG concentration up to 2 mM, it was possible to produce data sets which could be fitted to Michaelis-Menten kinetics using equation 1 (Figure 8, A and C). When the inverted data was analysed (Figure 8, B and D) very poor fits were achieved. When the same data were subjected to a global non-linear regression fit using commercial EnzFitter software it was clear that the data sets did not fit each other (Figure 9).

\[ V_i = \frac{V_{\text{max}}[S]}{K_{M,S} + [S]} \]  

(1)

where \( V_i \) = initial velocity, \( V_{\text{max}} \) = maximal velocity, \([S]\) = varied substrate (GABA, BAL or aKG), \( K_{M,S} \) = Michaelis constant for S. The other assays (SSADH and MSADC) were much more reliable, and produced data sets that could be fitted to

---

### Table 4: Activity (U mg⁻¹) of SkPyd4p, SkUga1p, ScUga1p and SpUga1p. Relative activities (%) are given in parentheses. The enzyme with the highest specific activity in each column is set to 100%.

<table>
<thead>
<tr>
<th></th>
<th>BAL*</th>
<th>GABA*</th>
<th>BAL***</th>
<th>GABA**</th>
</tr>
</thead>
<tbody>
<tr>
<td>SkPyd4p</td>
<td>4.5</td>
<td>1.6</td>
<td>3.52</td>
<td>0.75</td>
</tr>
<tr>
<td>SkUga1p</td>
<td>0.13</td>
<td>N.D.</td>
<td>0.10</td>
<td>12.8</td>
</tr>
<tr>
<td>ScUga1p</td>
<td>0.00</td>
<td>3.6</td>
<td>0.15</td>
<td>N.D.</td>
</tr>
<tr>
<td>SpUga1p</td>
<td>0.00</td>
<td>7.5</td>
<td>0.14</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* Determined with GDH assay. ** Determined with MSADC assay. *** Determined with SSADH assay. N.D. = not determined

### Kinetic analysis of SkPyd4p and SkUga1p

A full kinetic analysis on SkPyd4p and SkUga1p was done. Since SkUga1p had so little activity with BAL, only SkPyd4p was analyzed with BAL. A lot of effort was put into analysis using the GDH assay. At aKG concentration up to 2 mM, it was possible to produce data sets which could be fitted to Michaelis-Menten kinetics using equation 1 (Figure 8, A and C). When the inverted data was analysed (Figure 8, B and D) very poor fits were achieved. When the same data were subjected to a global non-linear regression fit using commercial EnzFitter software it was clear that the data sets did not fit each other (Figure 9).
EnzFitter algorithm (Figure 10, 11, 12). The GABA-AT data sets produced with the SSADH assay were fitted to equation 2.

\[ V_i = \frac{V_{\text{max}}[S1][S2]}{(K_{M,S1}[S2] + K_{M,S2}[S1] + [S1][S2])} \]  \hspace{1cm} (2)

where \( S1 = \text{GABA or BAL}, \ S2 = \text{aKG} \).

The BAL-AT data sets produced with the MSADC assay allowed usage of a higher concentrations of aKG (10 mM), revealing product inhibition by aKG. This data set was fitted to equation 3.

\[ V_i = \frac{V_{\text{max}}[S1][S2](1 + [S2]/K_{i,S2})}{(K_{M,S1}[S2] + [S1][S2])} \]  \hspace{1cm} (3)

where the inhibitor term \( 1+[S2]/K_{i,S2} \) has been added to eq. 2. affecting \( K_{M,S1} \) in a \( S2 \)-concentration dependent manor. The kinetic parameters for SkPyd4p and SkUga1p obtained from the fitting are presented in Table 5.

### Table 5: BAL-AT and GABA-AT analysis of SkPYD4 and SkUGA1.

<table>
<thead>
<tr>
<th></th>
<th>( V_{\text{max}} ) (U mg(^{-1}))</th>
<th>( K_{M,BAL} ) (mM)</th>
<th>( K_{M,GABA} ) (mM)</th>
<th>( K_{M,aKG} ) (mM)</th>
<th>( K_{i,aKG} ) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SkPyd4p GDH assay</td>
<td>7.47 \hspace{1cm} (1.32)</td>
<td>7.6 \hspace{1cm} (2.1)</td>
<td>N.D. \hspace{1cm}</td>
<td>2.1 \hspace{1cm} (0.6)</td>
<td>N.D. \hspace{1cm}</td>
</tr>
<tr>
<td>SkPyd4p SSADH assay</td>
<td>0.75 \hspace{1cm} (0.03)</td>
<td>N.D. \hspace{1cm}</td>
<td>1.8 \hspace{1cm} (0.2)</td>
<td>0.18 \hspace{1cm} (0.02)</td>
<td>N.D. \hspace{1cm}</td>
</tr>
<tr>
<td>SkUga1p SSADH assay</td>
<td>12.83 \hspace{1cm} (0.27)</td>
<td>N.D. \hspace{1cm}</td>
<td>3.2 \hspace{1cm} (0.2)</td>
<td>0.22 \hspace{1cm} (0.01)</td>
<td>N.D. \hspace{1cm}</td>
</tr>
<tr>
<td>SkPyd4p MSADC assay</td>
<td>6.43 \hspace{1cm} (0.21)</td>
<td>8.2 \hspace{1cm} (0.4)</td>
<td>N.D. \hspace{1cm}</td>
<td>2.9 \hspace{1cm} (0.2)</td>
<td>28.3 \hspace{1cm}</td>
</tr>
</tbody>
</table>

Standard errors of the fit are given in parentheses. One unit (U) is defined as the amount of enzyme needed to convert one \( \mu \)mole substrate into product per minute. N.D. = not determined.
Figure 8: Kinetic analysis of SkPyd4p BAL-AT activity (GDH assay). (A) Plot of activity (U mg⁻¹) vs. BAL concentration (mM). aKG concentrations: 0.25 mM (RED), 0.5 mM (CYAN), 1 mM (GREEN), 2 mM (BLUE). (B) Plot of activity (U mg⁻¹) vs. aKG concentration (mM). BAL concentrations: 2.5 mM (RED), 5 mM (CYAN), 10 mM (GREEN), 20 mM (BLUE), 40 mM (PINK). Curves in (A) and (B) are nonlinear regression fits of each data set to eq. 1. (C) Double reciprocal plot of activity (U mg⁻¹) vs. BAL concentration (mM). aKG concentrations: see (A). (D) Double reciprocal plot of activity (U mg⁻¹) vs. aKG concentration (mM). BAL concentrations: see (B).
**S. kluyveri PYD4 gene**

Figure 9: Full kinetic analysis of SkPyd4p BAL-AT activity (GDH assay). (A) Plot of activity (U mg⁻¹) vs. BAL concentration (mM). aKG concentrations: 0.25 mM (RED), 0.5 mM (CYAN), 1 mM (GREEN), 2 mM (BLUE). (B) Plot of activity (U mg⁻¹) vs. aKG concentration (mM). BAL concentrations: 2.5 mM (RED), 5 mM (CYAN), 10 mM (GREEN), 20 mM (BLUE), 40 mM (PINK). Curves in (A) and (B) are global nonlinear regression fits of all data set to eq. 2. (C) Double reciprocal plot of activity (U mg⁻¹) vs. BAL concentration (mM). aKG concentrations: see (A). (D) Double reciprocal plot of activity (U mg⁻¹) vs. aKG concentration (mM). BAL concentrations: see (B).
Figure 10: Full kinetic analysis SkPyd4p GABA-AT activity (SSADH assay). (A) Plot of activity (U mg\(^{-1}\)) vs. GABA concentration (mM). aKG concentrations: 0.1 mM (RED), 0.2 mM (CYAN), 0.4 mM (GREEN), 0.8 mM (BLUE), 1 mM (PINK). (B) Plot of activity (U mg\(^{-1}\)) vs. aKG concentration (mM). GABA concentrations: 1.5 mM (RED), 2.5 mM (CYAN), 5 mM (GREEN), 7.5 mM (BLUE). Curves in (A) and (B) are global nonlinear regression fits of all data set to eq. 2. (C) Double reciprocal plot of activity (U mg\(^{-1}\)) vs. GABA concentration (mM). aKG concentrations: see (A). (D) Double reciprocal plot of activity (U mg\(^{-1}\)) vs. aKG concentration (mM). GABA concentrations: see (B).
Figure 11: Full kinetic analysis of SkUga1p GABA-AT activity (SSADH assay). (A) Plot of activity (U mg⁻¹) vs. GABA concentration (mM). aKG concentrations: 0.1 mM (RED), 0.2 mM (CYAN), 0.4 mM (GREEN), 1 mM (BLUE), 2 mM (PINK). (B) Plot of activity (U mg⁻¹) vs. aKG concentration (mM). GABA concentrations: 2 mM (RED), 4 mM (CYAN), 10 mM (GREEN), 20 mM (BLUE), 40 mM (PINK). Curves in (A) and (B) are global nonlinear regression fits of all data sets to eq. 2. (C) Double reciprocal plot of activity (U mg⁻¹) vs. GABA concentration (mM). aKG concentrations: see (A). (D) Double reciprocal plot of activity (U mg⁻¹) vs. aKG concentration (mM). GABA concentrations: see (B).
Figure 12: Full kinetic analysis SkPyd4p BAL-AT activity (MSADC assay). (A) Plot of activity (U mg⁻¹) vs. BAL concentration (mM). aKG concentrations: 0.5 mM (RED), 1 mM (CYAN), 2 mM (GREEN), 5 mM (BLUE), 10 mM (PINK). (B) Plot of activity (U mg⁻¹) vs. aKG concentration (mM). BAL concentrations: 1 mM (RED), 2 mM (CYAN), 4 mM (GREEN), 8 mM (BLUE), 20 mM (PINK). Curves in (A) and (B) are global nonlinear regression fits of all data set to eq. 2. (C) Double reciprocal plot of activity (U mg⁻¹) vs. BAL concentration (mM). aKG concentrations: see (A). (D) Double reciprocal plot of activity (U mg⁻¹) vs. aKG concentration (mM). BAL concentrations: see (B).
DISCUSSION

Identification of a novel BAL-AT encoding gene

In *S. cerevisiae* only one gene encoding a GABA-AT (*ScUGA1*) is found, while *S. kluyveri* has two *ScUGA1*-like genes. This study identifies that one of these genes, *SkPYD4* is a novel GABA-AT-like encoding gene involved in BAL degradation. It was isolated from a *pyd4* mutant unable to grow on DHU, BUP and BAL as sole nitrogen source, but a strain with the gene disrupted (*Apyd4*) could grow on DHU and BUP as sole nitrogen source. This contradictory result is difficult to explain, since the *SkPYD4* gene complements all three growth defects. It could be that BAL and BAL derived compounds, which originate from DHU and BUP, are toxic to the *pyd4* strain. Close homologues of *SkPYD4* are found in two other yeast strains (*D. hansenii* and *C. albicans*), along with homologues of *PYD3*, but not *PYD2*. A more closely related species (*K. lactis*) does not have the *PYD4* gene, but has both a *PYD2* and a *PYD3* homologous gene. Apparently, the original *PYD4/UGA1* gene was duplicated in one yeast lineage, but later on in some descendant lineages one of the duplicated genes was lost. The estimated time point for the gene duplication would be after *Y. lipolitica* split from the *Saccharomyces/Candida/Debaryomyces* lineages, approx. >200 mill. years ago.

Gene duplication and speciation

The other homologous gene (*SkUGA1*) is shown to be equivalent to *ScUGA1*, both by phylogenetically relationship and by the phenotype of a targeted disruption. In mammals, BAL-AT and GABA-AT activity derive from the same gene-product. In rat (but not in humans or pig) post transcriptional modification in the liver transforms the brain type GABA-AT to the liver type BAL-AT I (Kontani et al., 1999). The naming of the two types of enzymes is a little misleading since the kinetic parameters reported in the literature are almost the same. The presence of two separate genetic loci in *S. kluyveri* for enzymes with distinct substrate specificity indicates a recent duplication of a BAL/GABA-AT into the specialized *PYD4* and *UGA1* gene(s). The function of the preduplicated gene can either be BAL-AT, GABA-AT, or both. To test this theory, *SkUga1p*, *SkPyd4p*, *ScUga1p* and *SpUga1p* were characterized. The *S. pombe*
homolog was believed to be an example of a preduplicated unspecialized gene, because it is the only homologous gene in the genome and phylogenetically it falls outside the SkUGA1 and SkPYD4 branch (Figure 2). The purified proteins clearly fell into two groups (Table 6). SpUga1p only had GABA-AT activity, which then suggests that the duplicated gene was likely a GABA-AT, and SkPYD4 is a neofunctionalization of one of the two copies of the yeast BAL-AT I (Force et al., 1999). The results with SpUga1p were a little surprising since S. pombe, like S. kluyveri, can use BAL as sole nitrogen source (pers. com. Jürgen Stolz, Dept. of Cell Biology and Plant Physiology, University of Regensburg, Germany). However, alternative routes for BAL/GABA are also found in Ustilago maydis, where a disruption of the ugatA gene encoding a Uga1p homologous protein only influences the BAL but not GABA utilization ability (Straffon et al., 1996).

Table 6: Summary of enzymatic activity of Pyd4p and Uga1p.

<table>
<thead>
<tr>
<th></th>
<th>BAL-AT</th>
<th>GABA-AT</th>
</tr>
</thead>
<tbody>
<tr>
<td>SkPyd4p</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SkUga1p</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>ScUga1p</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SpUga1p</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Characterization of SkPyd4p and SkUga1p

SkPyd4p is a PLP-dependent enzyme with the typical absorption maxima at 330 and 410 nm corresponding to the ketoenamine and enolimine form of the prosthetic group. Dialysis removed the prosthetic group completely, which confirms the binding capacity of PLP to the apoenzyme is not very strong. Addition of PLP to the apoenzyme can fully restore the activity of the holoenzyme.

The pH dependency of the absorption spectrum indicates that the internal aldimine is present in both the ketoenamine and enolimine form.

Both SkPyd4p and ScUga1p show a reaction mechanism involving two half-reactions (8 and 9 respectively). In the first half-reaction, the amino-group is transferred from BAL or GABA to the internal aldimine (PLP), forming a semialdehyde and pyridoxamine 5’-phosphate (PMP). In the second half-reaction, the amino-group of PMP is moved to aKG, forming glutamate and restoring PLP.
S. kluveri PYD4 gene

\[
\text{BAL/GABA + E-PLP} \quad \leftrightarrow \quad \text{MSA/SSA + E-PMP} \tag{8}
\]

\[
\text{E-PMP + aKG} \quad \leftrightarrow \quad \text{E-PLP + glutamate} \tag{9}
\]

This ping-pong mechanism is evidenced by the parallel lines in the double-reciprocal Lineweaver-Burk plots. It was further found that at high aKG concentrations (10 mM) in the SkPyd4p (MSADC) experiments, a larger slope was observed when BAL was the varied substrate, and upwardly curving data, when aKG was the varied substrate. The latter is due to competitive binding of aKG to the E-PLP enzyme species. The inhibition constant of SkPyd4p for aKG (\(K_{i,aKG}\)) of 28.3 mM is almost three times as high as that of \(E. coli\) GABA-AT with a \(K_{i,aKG}\) of 10.2 mM (LIU 2005). This could be a consequence of the neofunctionalization of SkPYD4 into a BAL-AT, where the active site could be modified in a way that makes GABA (and aKG) less likely to bind to the E-PLP species.

It seems though that the affinity (\(K_m\)) for GABA is better in SkPyd4p than in SkUga1p (1.8 mM and 3.2 mM, respectively), but the real difference is in the catalytic efficiency. SkUga1p is 10-times more efficient than SkPyd4p (\(V_{max}/K_m\) of 4.0 versus 0.4 U mg\(^{-1}\) mM\(^{-1}\), respectively. More detailed questions about substrate specificity and structure/function relationship will be investigated when the structure determination of SkPyd4p and SkUga1p is completed.

**ACKNOWLEDGEMENTS**

I would specifically like to acknowledge the following two people for their active participation in parts of this work presented here.

Prof. Klaus Schnackerz, University of Würzburg, Germany, for helping with purification, characterization and optimization of the kinetics of the enzymes.

Birgit Andersen, lab technician from the lab, for helping with purification.
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CHAPTER 6

GENERAL DISCUSSION

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Chapter 6

OUTLINE

When this project started, one of the goals was to explain the genetic and enzymatic background of pyrimidine degradation (PYD genes) in the yeast, *Saccharomyces kluyveri*, and to study the origin of this pathway. It was believed that the pathway would be similar to the reductive pathway found in mammals, insects and bacteria, and that *S. kluyveri* could be used as a model to understand this pathway in any eukaryote. The two genes, *PYD2* and *PYD3*, involved in this pathway, had already been characterized (Gojkovic et al., 2000; Gojkovic et al., 2001). The previous isolation of *pyd2* and *pyd3* mutants, both showing a clear genotype – phenotype relationship, e.g. both unable to grow on uracil, was very supportive towards the presence of an intact reductive pathway. So, in principle it was only the first piece of the puzzle, *PYD1*, coding for dihydropyrimidine dehydrogenase (DHPDH) catalyzing the reduction of uracil to dihydrouracil (DHU), that was missing. Besides its ability to utilize exogenous uracil, DHU and beta-ureidopropionate (BUP) as sole nitrogen source, *S. kluyveri* can also utilize exogenous beta-alanine (BAL), which is a product of the Pyd3p enzyme activity. Mutants in *S. kluyveri* unable to utilize BAL as nitrogen source (*bac1*) had also been identified previously (Gojkovic et al., 1998). Another goal of this project was to study the genetic and enzymatic basis of *S. kluyveri*’s ability to grow on BAL as nitrogen source.

MY THREE YEARS IN THE LAB

At the beginning it looked like a clear genotype - phenotype relationship between the presence of an active uracil reductive catabolic pathway and the ability to use intermediates of this pathway as sole nitrogen source (Chapter 3 and (LaRue and Spencer, 1968)). The ability to use uracil, DHU and BUP as nitrogen sources followed each other in most of the yeast species tested, which supports the existence of a linked pathway. A clear correlation between the loss of the ability to use uracil, DHU and BUP as sole nitrogen sources and the recent whole genome duplication was observed (Chapter 3). However, some of the tested yeast did not follow the path, like for example species of *Torulaspora* and *Hanseniaspora* (Chapter 3, Table 1). Another thing was, when the thirty-eight strains were tested; the loss of the ability to use BAL as nitrogen source was not linked to the whole genome duplication, but apparently
lost more or less randomly in several lineages (Chapter 3, Table 1). The genotype – phenotype relationship seen in Chapter 3, was not supported when more direct genetic methods were used (Chapter 4). Firstly, following an extensive mutagenesis of *S. kluyveri* strains, the screening was performed to find strains unable to grow on uracil as sole nitrogen source. When the novel mutants (*pyd*) were analysed, a very surprising picture emerged. The organisms ability to utilize uracil, DHU, BUP and BAL as sole nitrogen source has for decades been used as indication of an active reductive catabolic pathway (LaRue and Spencer, 1968). When screening for mutants, it is simply more convenient to make growth experiments rather than to purify and characterize specific enzymatic activities. In this respect, one crucial fact was totally neglected, *S. kluyveri* grows very weakly with thymine as sole nitrogen source, even though the intermediates D-dihydrothymine (DHT), D-beta-ureidoisobuturate (DBUIB) and D-beta-aminoisobutyrate (DBAIB) all serve as good nitrogen sources. In bacteria, shown to possess either the reductive pathway (West, 2001) or oxidative pathway (Soong et al., 2001), thymine and uracil are equally good as a nitrogen source and as inducers of all the enzymes involved. The obtained *pyd* mutants could be divided into six groups, based on interallelic complementation, but none of the mutants were allelic with *PYD2* or *PYD3*. All the *pyd* mutants could grow on DHU, BUP and BAL. When the *PYD2* and *PYD3* loci were disrupted, the resulting strains could grow on uracil as nitrogen source, showing the presence of two pathways, one for uracil and one for DHU degradation. The mutant loci of the uracil degradation were identified and the metabolic intermediates of the pathway were examined using HPLC (Chapter 4).

Previous attempts to isolate the gene(s) involved in BAL catabolism were hindered by the *bac1* mutants inability to be complemented by the *S. kluyveri* genomic library. The isolation of the gene *PYD4* encoding a BAL aminotransferase (BAL-AT) did not come from a mutant with the same phenotype as the *bac1* mutants. The gene was isolated from the *pyd4* mutant Y947 which, unlike *bac1* mutants, could not utilize exogenous DHU and BUP as sole nitrogen sources (Chapter 5, Table 2). The SkPyd4p was found to be a classical PLP-dependent enzyme, which uses BAL or gamma-aminobutyrate (GABA) as nitrogen donor and alpha-ketoglutarate (aKG), but not pyruvate as nitrogen acceptor. When SkPyd4p was compared with SkUga1p, ScUga1p and SpUga1p, it was found that while the latter enzymes all were highly
specific GABA-AT enzymes, SkPyd4p was a more broad and less efficient BAL/GABA-AT enzyme.

GENETIC SPLIT OF URACIL AND DHU PATHWAYS

Six loci were found to be involved in uracil degradation in *S. kluyveri*. They were termed *PYD11,12,13,14,15,16* (Chapter 4) each complementing a specific *pyd* mutant. All *PYD1X* could grow on DHU as a sole nitrogen source, meaning either an extremely unlucky distribution of mutations in the genome obtained through EMS mutagenesis, or uracil and DHU degradation could be genetically and biochemically separated. Disruption of the previously characterized *PYD2* and *PYD3* loci, showed that Δ*pyd2* and Δ*pyd3* can grow on uracil, indicating that the *pyd2* and *pyd3* strains, which were previously isolated, were likely double mutants (containing also a *pyd1X* mutation). Another example of geno-/phenotype controversy was the isolation of the *pyd4* mutant strain Y947 (Chapter 5). This strain was isolated by chance, when selecting for mutants unable to grow on DHU. However, a *pyd4* genotype was expected to be able to grow on DHU (and BUP) as sole nitrogen source, because of the nitrogen liberated from the beta-ureidopropionase (UP) reaction. When the gene was disrupted, the resulting Δ*pyd4* strain could grow (as expected) on DHU and BUP. An explanation could be that Pyd4p is a negative regulator of *PYD2* and *PYD3* when BAL is present at high concentrations, and that the *pyd4* mutant locus produces a protein, which cannot catabolize BAL, but can be involved in negative regulation. When wild type *PYD4* is introduced, it removes BAL, hereby restoring the whole pathway. In the Δ*pyd4* strain the negative regulation is absent, therefore *PYD2* and *PYD3* are not regulated by high internal BAL concentration. *S. kluyveri* mutant, having the same phenotype as Δ*pyd4*, were isolated (*bac1* mutants) previously by EMS mutagenesis (Gojkovic et al., 1998), but these mutants could not be complemented by the genomic library or the isolated *PYD4* containing plasmid (data not shown). These findings on "false" phenotypes strongly motivated the use of targeted disruption in the further studies to make sure that each phenotype is assigned the right genotype. Disruption was made of all *PYD1X* loci and the resulting mutants could not grow on uracil as sole nitrogen source (Chapter 4, Table 6). The Δ*pyd16* strain could not use either uracil or uridine as sole nitrogen source, while the original
Y811 (pyd16) grew well on uridine. Again, a discrepancy which could be due to a leaky mutation or presence of several additional mutations or a pleiotropic effect of a single mutation. Since Y811 is also ura3 it would be termed a synthetic lethal, since it cannot make pyrimidines de novo (ura3) and it cannot salvage pyrimidines (pyd16 is identical to fur1). Interestingly, when FUR1 was originally characterized, two recessive mutants were found in the same allele (fur1-1 and fur1-6) (Jund and Lacroute, 1970). The only difference was that fur1-1 mutants were as resistant to 5-fluorouridine as the uridine transport mutants (fui1), while fur1-6 had wild type sensitivity. This could indicate that PYD16/FUR1 is also involved in uridine utilization/transport in general. While the pyd16 strain as mentioned is also ura3, the ∆pyd16 is not, but such a strain has been constructed in S. cerevisae and therefore shown to be non-lethal (Seron et al., 1999). If utilization of uracil and uridine is blocked, then it must be exogenous cytidine (not cytosine), that serves as sole pyrimidine source in this strain (and maybe some unspecific transport of nucleotides).

MASKING THE TWO PATHWAYS

When the ability to utilize uracil, DHU and BUP was examined in different yeasts (Chapter 3), it seemed that the traits are ”linked”. Either a yeast can use none or all of the intermediates. This pattern is expected from intermediates belonging to a single pathway and not from two independent pathways. Apparently, the major metabolic rearrangements, that occured after the last whole genome duplication in yeast (Cliften et al., 2006), masked the split in the pathways, and both became redundant. Off course, this leads to speculations on the origin of the DHU degradation pathway. In nature, DHU comes from the reduction of uracil, either as an intermediate in the catabolic pathway or as a constituent of tRNA, where a special uracil gets reduced directly on the RNA molecule. All tRNAs isolated from S. cerevisiae have such a modification, and this modification is achieved by the DUS gene family encoding dihydouridine synthases (Xing et al., 2004). The further fate of dihydouracil from tRNA is not known, but it seems very unlikely, that S. kluveri PYD2 and PYD3 should be involved in this alone. Dihydouracil could also originate from damaged DNA, where more or less efficient systems are involved in their removal (Venkhataraman et al., 2001). Yet another explanation could be the presence of DHU
in *S. kluveri* natural habitats, e.g. fruit flies (*Drosophila* species) or trees (Barker and Miller, 1969).

**A NEW PATHWAY**

Deciphering the function of each *PYDIX* gene in the new pathway is hindered by the fact, that very little is known about any of the intermediates except the starting molecule, uracil, UMP and the almost final product, urea. The function of *PYD12*, *PYD13*, *PYD15* and *PYD16* was determined by homology searches, while function of *PYD11* and *PYD14* had to be speculated on. Eventually, a plausible pathway was constructed, giving an explanation for the role of all *PYDIX* genes found (Figure 1) (see also Chapter 4).

**First step (**PYDI6**)**
The finding that *pyd16* could grow on uridine, leads to the hypothesis, that a uracil nucleoside or nucleotide species is the first actual reaction intermediate. And with the disruption of uridine hydrolase gene (*URH1*) further evidence came, when the strain could still grow on uridine (and uracil) as sole nitrogen source. Apparently, uridine did not have to be changed into uracil, in order for the nitrogen to be extracted. This gave a strong indication that uridine or a phosphorylated product (UMP, UDP and UTP) is the starting and central point for the degradation, and that the first step in this pathway is equal to the first step in the salvage pathway of uracil (Reaction 1).

\[
\text{uracil} + \text{PRPP} \leftrightarrow \text{UMP} + \text{pyrophosphate}
\]

**Second step (**PYDI1**)**
Pyd11p shares a common motif with GTP cyclohydrolase II (GCH2), which catalyses the first step in the biosynthesis of riboflavin. GCH2 uses GTP as substrate, by first covalently attaching GMP to the enzyme (releasing pyrophosphate), and then hydrolytic opening of a guanine ring. The structure of *E. coli* GCH2 was solved recently and shows a GTP analog bound in the active site with a Mg\(^{2+}\) and a Zn\(^{2+}\) atom pr. monomer (Ren et al., 2005). Pyd11p has a GCH2 motif, with many residues
Figure 1: Proposed novel uracil degradation pathway. Firstly, uracil enters the cell and gets phosphoribosylated to UMP by Pyd16p. The entry point for the degradation pathway could be any of uridine, UMP, UDP or UTP (UriX). The usual cell enzymes convert UMP to UriX. UTP seems to be the most likely candidate as entry point, but the other uridine species are also possible. UriX is hydrolyzed by Pyd11p, and the product ureidomalonic semialdehyde–ribose–5-monophosphate (UMSA-RX) is released. Unspecific or enzymatic hydrolysis splits the N-glucosidic bond of UMSA-RX to UMSA and Rib-X. Pyd14p hydrolyzes UMSA to urea and malonic semialdehyde (MSA). Urea gets degraded to ammonia and carbon dioxide in a two step reaction by the enzymatic reactions of Pyd13p and Pyd15p. The further fate of MSA is not known, but conversion to BAL through Pyd4p is a possibility. Pyd5p reaction could involve coupling to CoA (MSA dehydrogenase), oxidation (malonate dehydrogenase) or decarboxylating and reduction (MSA decarboxylase and alcohol dehydrogenase).

conserved (Figure 2). These conserved residues are located in the active site, especially around the ribose and phosphate binding, but also two residues flanking each side of the guanine ring (Figure 3). At the same time the two residues, hydrogen-binding with the pyrimidine part of the guanine ring, are different in Pyd11p. The residue believed to be involved in the first nucleophile attack on GTP in Ec GCH2, Arg128 is also conserved in all SkPyd11p-like proteins (Figure 2, residue 98).
### Figure 2: Sequence alignment of Pyd11 and GCH2 proteins.

Only residues homologous to the Ec GCH2 R49 – K154 are shown. Comments are *
= conserved and in substrate binding in Ec GCH2, 
= conserved and not involved in substrate binding in Ec GCH2, \( \times \) = not conserved and involved in substrate binding in Ec GCH2.

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**Comment:** * = conserved and in substrate binding in Ec GCH2, \( \# \) = conserved and not involved in substrate binding in Ec GCH2, \( \times \) = not conserved and involved in substrate binding in Ec GCH2.
Substitution of the two non-conserved active site residues would predict a much smaller active site, making it impossible for a guanine ring to fit inside (Figure 3). It is possible that an uracil ring would fit in this active site indicating that Pyd11p uses a ribosylated uracil species (UriX) as substrate. The best candidate seems to be UTP, because the residues involved in stabilizing/binding the ribose-triphosphate part of GTP is conserved. The specific nucleophile, arginine, as mentioned above is also conserved, which indicates a similar reaction mechanism, with covalently bound UMP in the case of Pyd11p reaction.

If cell free extracts from *S. kluyveri* cells pre-grown in media containing uracil as sole nitrogen source, are incubated with uridine, UMP, UDP or UTP, no decline in absorbance at 260 nm is seen (data not shown). In these cases, a decline would be expected from a cyclohydrolase reaction, but even though none is seen, the reaction could be dependent on specific co-factors or buffer conditions. Overexpression, purification and characterization of SkPyd11p would help in the identification of the
substrates (under investigation). The novel compounds, UMSA-RX and UMSA, are not found in the literature, but a similar compound (ureidomalonate, UMAL) is an intermediate in the oxidative degradation of uracil. UMAL gets hydrolysed to urea and malonate by ureidomalonase (Soong et al., 2001). Since urea should be formed at a latter point, the N-glucosidic bond between the ureido group and the ribose needs to be hydrolysed. This could happen in in-vivo by some unspecific reaction (enzymatic or non-enzymatic).

Third step (PYD14):
Pyd14 was the only protein which had no homology to any known protein motifs. This makes it a candidate as a novel enzymatic catalyst, which use the uncharacterized UMSA as substrate. The proposed mechanism for Pyd14p is to hydrolyse UMSA to yeild urea and MSA (Figure 1).

Fourth, fifth step (PYD13,15):
It seems that the 1-N, 2-C and 3-N atoms eventually become urea and then through the carboxylase and hydrolase activity of Pyd13,15p get mineralized to ammonia and carbon dioxide. Urea formation from uracil degradation has been reported or hypothezised a number of times (Di Carlo et al., 1952; Reinbothe, 1964; Thwaites et al., 1979), and was also found in extracts from both, pyd13 and pyd15 mutants. Not all steps from the entry of uracil into the cells to the final ammonia are accounted for by the action of the PYD1X genes. The rest of the genes must either have been missed by the mutagenesis and screening procedure or be essential in normal cell metabolism (and a mutation in the gene would be lethal).

Regulation (PYD12)
Pyd12p has strong homology to zinc-finger transcription factors from fungi, which strongly indicates a regulatory, rather than an enzymatic role of this protein (Todd and Andrianopoulos, 1997). The function of these proteins is to regulate gene expression either positively or negatively, this would highly likely be Pyd12p’s function. S. cerevisiae has a protein (YDR520Cp) which is homologous to Pyd12p, which indicates that these proteins have a similar function in both S. cerevisiae and S. kluyveri. Since S. cerevisiae does not have homologs of Pyd11p and Pyd14p (and
cannot use uracil as sole nitrogen source), the function of YDR520Cp/Pyd12p could be to regulate pyrimidine metabolism in a more general fashion, including both, biosynthetic and salvage pathways.

WHAT IS THE FUNCTION OF THE URACIL PATHWAY?

The organisms (presented in Chapter 4), which have the PYD11 and PYD14 genes, may represent only a fraction of the organisms having the ”new” degradation pathway. This pathway was lost in the yeast lineage which underwent the whole genome duplication event, but it remained to be present in many, if not all, other fungi (see Appendix). Therefore, it must play an important role in the fungal metabolism. From the deduced pathway uracil is catabolized to urea and MSA, but it needs to go to the ribosylated state, UriX, using at least one PRPP molecule in the process. Why ”waste” energy to get urea and MSA? If the environment has limited nitrogen sources, then the ability to degrade any exogenous nitrogen-containing compounds is an advantage. Pyrimidines are widely distributed in nature, and might be a dominant nitrogen source in some places where yeast live. The ”novel” pathway would likely be under control of the nitrogen catabolite repression system and be induced by uracil and uridine. Another function could be regulation of the intracellular pyrimidine nucleotide pool under nitrogen-limiting conditions. Break-down of pyrimidines might help the cells adapt to a slower growth rate when going from rich to poor conditions. The presence of a reductive uracil catabolism in plants (Katahira and Ashihara, 2002) also argues that the progenitor of fungi likely had the first reductive uracil degradation step (PYD1). Apparently, while the ”novel” pathway (through UriX to urea) evolved during the evolutionary history, the uracil to DHU step was lost. However, could there remain any traces of the ”novel” pathway in plants?

If MSA is really an intermediate, then the possibility of producing BAL from a reverse BAL-AT (Pyd4p) reaction, could also be one of the functions. In S. cerevisiae, BAL is produced from polyamine break-down, while for example S. pombe cannot generate BAL in this way. Instead, S. pombe can convert BUP and uracil, (but not DHU) to BAL, as was shown in a mutant unable to import exogenous pantothenate (Stolz et al., 2004). If BAL comes from uracil in S. pombe it could then explain the presence of Pyd11p and Pyd14p, and the pathway could look like the proposed one
(Figure 1). Even though the SpUga1p enzyme is a specific GABA-AT, with very little BAL-AT activity (Chapter 5), the reverse reaction might be enough to supply the cells with BAL from MSA, as proposed for *A. nidulans* (Arst, Jr., 1978). There might also be a specific BAL-AT protein in *S. pombe*, not homologous to Uga1p or Pyd4p, since *S. pombe* can use BAL as sole nitrogen source. A BLAST search in the *S. kluyveri* genome sequence at NCBI showed that 9 of 11 *S. cerevisiae* proteins involved in pantothenate biosynthesis are present. The two missing genes were a second copy of *ALD2*/*ALD3* (which is known to be a recent duplication), and *ECM31*, which is involved in the (R)-pantoate synthesis. It could be that *ECM31* is present in the part of the *S. kluyveri* genome not sequenced (<5%). All the homologous proteins indicate an intact pantothenate pathway with BAL supplied from the polyamine breakdown. If the novel uracil degradation pathway can produce BAL, then *S. kluyveri* would seem to have an overflow of possibilities to make BAL (Figure 4).

**DUPLICATION AND SPECIATION OF UGA1/PYD4 GENES**

Duplications are one of the main sources of new genes. While the majority of duplicated copies, sooner or later are lost from the genome, both copies can be preserved if they develop a different expression pattern (regulation) or they divide the original function between them or one of the copies develop a new function. Mammals only have one gene encoding a GABA-AT with both BAL-AT I and GABA-AT activities. A post transcriptional modification process modifies the enzyme into a liver type BAL-AT I and a brain type enzyme GABA-AT (Kontani et al., 1999). This maturation causes a minor but significant change in the enzyme affinity regarding BAL (K<sub>M,BAL</sub> = 5.3 mM and 6.1 mM, V<sub>max</sub> = 0.83 U mg<sup>-1</sup> and 1.00 U mg<sup>-1</sup> for liver type BAL-AT I and brain type GABA-AT respectively), which could have a metabolic influence. In *S. kluyveri* these two functions are split between two genes. One, *SkUGA1*, encodes a specialized GABA-AT, which has high homology to UGA1 from *S. cerevisiae*, and is needed for normal utilization of GABA as sole nitrogen source. The other gene, *SkPYD4*, encodes a non-specialized GABA/BAL-AT, which is absent in the *S. cerevisiae* genome. The difference in substrate
specificity can be seen from targeted gene disruption of the two loci. The \textit{PYD4} disruption causes a cell to grow very weakly on BAL as sole nitrogen source, while \textit{UGA1} disruption still results in moderate growth on GABA as sole nitrogen source (Chapter 5, Table 2). Double disruption results in very weak growth on both substrates. This shows that SkPyd4p is the only specialized BAL catabolizing enzyme in \textit{S. kluyveri}, while GABA gets metabolized primarily by SkUga1p, and to some degree by SkPyd4p. The weak background growth, could be a result of unspecific AT-activity from some of the other AT in the cells.

Homologs of the \textit{UGA1} gene are found in all annotated fungi species from the NCBI databank, so its function seems to be preserved. But what is the reason that the \textit{UGA1} gene is present in fungi? GABA comes from decarboxylation of glutamate and gets metabolised into succinate and thereby enters the TCA cycle. The best explanation for preservation of this pathway should be natural sources of GABA in the fungal habitats.
From the phylogeny of the Uga1/Pyd4 enzymes it is deduced that the duplication into Uga1p and Pyd4p took place after *Y. lipolytica* branched out. However, in the fungal group both *G. zeae* and *C. neoformans* have two Uga1p encoding genes (Chapter 5, Figure 2). In the basidiomycete *Ustilago maydis* only one gene is present with homology to *UGA1* from *S. cerevisiae* (Straffon et al., 1996). Interestingly enough the gene is induced by both GABA and BAL, but disruption of the gene only alters the BAL utilization (five fold decrease in growth rate on BAL as nitrogen source) and not GABA utilization (same growth rate as parent strain) (Straffon et al., 1996). *U. maydis* Uga1p (XP_757227) groups phylogenetically with the SpUga1p characterized to be a specific GABA-AT. Apparently, omega-acid transamination is greatly shrouded by the over-lapping specificities from other transaminases, and it is difficult to rely only on genetical data, but the enzyme activity needs to be measured on purified enzymes. While most other yeast have only one gene (*UGA1*), why does *S. kluyveri* need two? It could simply be to achieve fine tuning of utilization of different nitrogen sources.

**SPECULATIONS ON THE PYD5 MEDIATED REACTION**

Since *S. kluyveri* (and many other species) can utilize BAL as sole nitrogen source, an efficient system for metabolising BAL and the product MSA, needs to be present, too. In mammals and bacteria, MSA is converted to acetyl-CoA by methylmalonic semaldehyde dehydrogenase (MMSADH), normally associated with valine metabolism (Goodwin et al., 1989; Zhang et al., 1996). In the soil bacterium *P. pavonaceae* 170 a MSA decarboxylase (MSAD) was recently identified as one of the enzymes involved in trans-1,3-dichloropropene catabolism (Poelarends et al., 2003). MSAD converts MSA into acetaldehyde and CO$_2$ (note that this enzymatic reaction was utilized in BAL-AT assay in Chapter 5) and the acetaldehyde could get reduced to ethanol by alcohol dehydrogenase. Homologs of MMSADH or MSAD are not found in the *S. kluyveri* genome sequence. Another possibility would be oxidation of MSA to malonate by a MSA dehydrogenase in a reaction similar to SSA to succinate by SSADH (Figure 5). There are two *S. kluyveri ScUGA2* homologous genes in the genome nucleotide sequence at NCBI (termed *UGA2a* and termed *UGA2b* with the
A BLAST search of ScUga2p identified many homologs in all fungi, and some had, like *S. kluyveri*, two genes. A phylogenetic tree of Pyd4/Uga1 proteins and Uga2 proteins in yeast shows that the *UGA2* gene was duplicated at approximately the same time as the *PYD4/UGA2* ancestor gene, and that both pairs likely evolved in parallel (Figure 6).

**Figure 5:** GABA and BAL pathways. GABA gets metabolized first to succinic semialdehyde (SSA) by Uga1p (GABA-AT) and then SSA gets oxidized to succinate by Uga2p (SSA dehydrogenase). BAL could be metabolized in a similar fashion with first product being MSA by the action of Pyd4p (BAL-AT) and then MSA gets oxidized by Pyd5p (MSA dehydrogenase).
Figure 6: Phylogenetic trees of Uga1 (left) and Uga2 (right) proteins in yeast. Left: The split into Uga1p and Pyd4p group is seen, and Y. lipolytica Uga1p being the closest to a preduplication form. Right: The split into Uga2p (S. cerevisiae-like) and Pyd5p (putative MSA dehydrogenase) group is seen, and Y. lipolytica Uga2p being the closest to a preduplication form. In both trees, S. pombe proteins (SpUga1p and SpUga2p) were used as an outgroup.
CONCLUDING REMARKS

The work presented here opens a whole new area of research. The genetic foundation for a new pathway has opened interesting questions to be answered. What are the actual intermediates? This would definitely help solving the question as to what the function is. A putative gene regulating protein was identified (Pyd12p), but its role is difficult to deduce, since even though the rest of the pathway got redundant after the whole genome duplication, Pyd12p did not (and is present in S. cerevisiae). Microarray for S. kluyveri are available now, and could prove a valuable tool to identify even further genes involved in uracil and BAL degradation.

Note added in proof: Just recently a PNAS paper reported that operon b1012 in E. coli K12 is involved in a "novel" pyrimidine degradation pathway (Loh et al., 2006). The operon is composed of seven unidentified ORF’s (none with homology to any of PYDIX genes presented in this thesis) and the end-products were determined to be 3-hydroxypropionate, ammonia and carbon dioxide. Urea was not an intermediate.

REFERENCES


CHAPTER 6

APPENDIX

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LIST OF ORGANISMS

Organisms were selected based on a BLAST search for the presence of PYD11 and PYD14 homologous genes using TBLASTN into translated nucleotide database at NCBI. If only one of the genes was found this is mentioned after the species name (in the brackets). In the fungi group all 42 strains are shown.

**Bacteria having PYD11 and PYD14 (of 545 genomes)**

1. Proteobacteria α Bradyrhizobium japonicum
2. Proteobacteria α Bradyrhizobium sp. BTAi1
3. Proteobacteria β Polaromonas
4. Proteobacteria β Ralstonia eutropha
5. Proteobacteria β Ralstonia metallidurans
6. Proteobacteria γ Legionella pneumophila str. Lens
7. Proteobacteria γ Legionella pneumophila str. Paris
8. Proteobacteria γ Legionella pneumophila str. Philadelphia 1
9. Proteobacteria δ/ε Bdellovibrio bacteriovorus
10. Cyanobacteria Synechococcus sp. JA-2-3B’a(2-13)
11. Actinobacteria Mycobacterium vanbaalenii PYR-1
12. Actinobacteria Mycobacterium flavescens PYR-GCK

**Eukaryota (diatoms)**

1. Bacillariophyta Coscinodiscophyceae Thalassiosira pseudonana (PYD11 only)
Fungi having *PYD11* and *PYD14* (of 42 genomes)

1. Ajellomyces capsulatus
2. Aspergillus clavatus
3. Aspergillus flavus
4. Aspergillus fumigatus
5. Aspergillus nidulans
6. Aspergillus terreus
7. Botryotinia fuckeliana
8. Chaetomium globosum
9. Coccidioides immitis
10. Gibberella moniliformis
11. Gibberella zeae
12. Magnaporthe grisea
13. Neosartorya fischeri
14. Neurospora crassa
15. Phaeosphaeria nodorum
16. Sclerotinia sclerotiorum
17. Trichoderma reesei
18. Uncinocarpus reesii
19. Eremothecium gossypii
20. Kluyveromyces lactis
21. Kluyveromyces waltii
22. Saccharomyces kluveri
23. Yarrowia lipolytica
24. Schizosaccharomyces pombe
25. Coprinopsis cinerea okayama
26. Cryptococcus neoformansvar.neoformans
27. Phanerochaete chrysosporium
28. Ustilago maydis
29. Rhizopus oryzae

Fungi not having *PYD11* and *PYD14* (of 42 genomes)

1. Candida albicans
2. Candida glabrata
3. Candida tropicalis
4. Clavispora lusitaniae
5. Debaryomyces hansenii
6. Pichia guilliermondii
7. Saccharomyces bayanus
8. Saccharomyces castellii
9. Saccharomyces cerevisiae
10. Saccharomyces kudriavzevii
11. Saccharomyces mikatae
12. Saccharomyces paradoxus
13. Encephalitozoon cuniculi