# Eukaryotic β-Alanine Synthases Are Functionally Related but Have a High Degree of Structural Diversity

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ABSTRACT

β-Alanine synthase (EC 3.5.1.6), which catalyzes the final step of pyrimidine catabolism, has only been characterized in mammals. A *Saccharomyces kluyveri pyd3* mutant that is unable to grow on *N*-carbamyl-β-alanine as the sole nitrogen source and exhibits diminished β-alanine synthase activity was used to clone analogous genes from different eukaryotes. Putative *PYD3* sequences from the yeast *S. kluyveri*, the slime mold *Dictyostelium discoideum*, and the fruit fly *Drosophila melanogaster* complemented the *pyd3* defect. When the *S. kluyveri PYD3* gene was expressed in *S. cerevisiae*, which has no pyrimidine catabolic pathway, it enabled growth on *N*-carbamyl-β-alanine as the sole nitrogen source. The *D. discoideum* and *D. melanogaster PYD3* gene products are similar to mammalian β-alanine synthases. In contrast, the *S. kluyveri* protein is quite different from these and more similar to bacterial *N*-carbamyl amidohydrolases. All three β-alanine synthases are to some degree related to various aspartate transcarbamylases, which catalyze the second step of the *de novo* pyrimidine biosynthetic pathway. *PYD3* expression in yeast seems to be inducible by dihydrouracil and *N*-carbamyl-β-alanine, but not by uracil. This work establishes *S. kluyveri* as a model organism for studying pyrimidine degradation and β-alanine production in eukaryotes.

CELLS constantly build up and break down nucleotide compounds to ensure a balanced supply of nucleotides for nucleic acid synthesis. The catabolic pathway, together with the *de novo* biosynthetic and salvage pathways, determines the size of the pyrimidine pool in the cell. In the first step of pyrimidine degradation, dihydropyrimidine dehydrogenase (EC 1.3.1.2) reduces uracil and thymine to the corresponding 5,6-dihydro derivatives (Figure 1). Thereafter, dihydropyrimidinase (EC 3.5.2.2) opens the pyrimidine ring and, depending on the dihydropyrimidine, forms *N*-carbamyl-β-alanine or *N*-carbamyl-β-aminoisobutyric acid, which is degraded to the corresponding β-amino acids (Wallach and Grisolia 1957; Figure 1).

*N*-Carbamyl-β-alanine amidohydrolase (EC 3.5.1.6, also known as β-alanine synthase or β-ureidopropionase) catalyzes the third and final step of pyrimidine degradation: irreversible hydrolysis of *N*-carbamyl-β-alanine to β-alanine (Caravaca and Grisolia 1958). This step of pyrimidine catabolism is poorly understood. In bacteria this enzyme enables utilization of a variety of carbamyl amino acids (Watabe *et al.* 1992; Ogawa and Shimizu 1994; Ikenaka *et al.* 1998; Nanba *et al.* 1998). The eukaryotic counterpart has been purified from calf (Waldmann and Schnackerz 1989), rat (Tamaki *et al.* 1987), mouse (Sanno *et al.* 1970), and *Euglena gracilis* (Wasternack *et al.* 1979). Nevertheless, so far the only known eukaryotic β-alanine synthase genes are the ones isolated from rat (Kvalnes-Krick and Traut 1993) and human (Vreken *et al.* 1999). The rat enzyme, depending on the presence of allosteric effectors, exists as a stable homohexamer, inactive trimer, or active homododecamer (Matthews and Traut 1987). The native hexameric enzyme occurs in the absence of ligands, but readily dissociates to trimers in response to β-alanine, or associates to form dodecamers upon binding of the substrate. A model suggesting an allosteric regulatory site distinct from the catalytic site was proposed (Matthews *et al.* 1992). The regulation of the mammalian genes encoding β-alanine synthase was not studied.

Degradation of uracil is the only pathway providing β-alanine in animal tissues (Traut and Jones 1996). Microorganisms may additionally form β-alanine either by direct α-decarboxylation of L-aspartate (Williamson and Brown 1979) or by degradation of polyamines (Large 1992). β-Alanine is an indispensable metabolite as it precedes formation of coenzymeA (CoA) and pantothenic acid in bacteria and fungi (Cronan *et al.* 1982). This unusual amino acid also plays a role in pigmentation of insect cuticle (Jacobs 1980) and fungal cell walls (Jacobs 1982), while in mammals and birds it is involved in the formation of neurally active dipeptides (anserine and carnosine). From a clinical point of view, β-alanine and its derivatives represent degradation products of one of the most employed anti-cancer drugs, 5-fluorouracil, and may be responsible for the neurotoxicity and brain necrosis observed during chemotherapy (Okeda *et al.* 1990). Because of its chemical similarity to the

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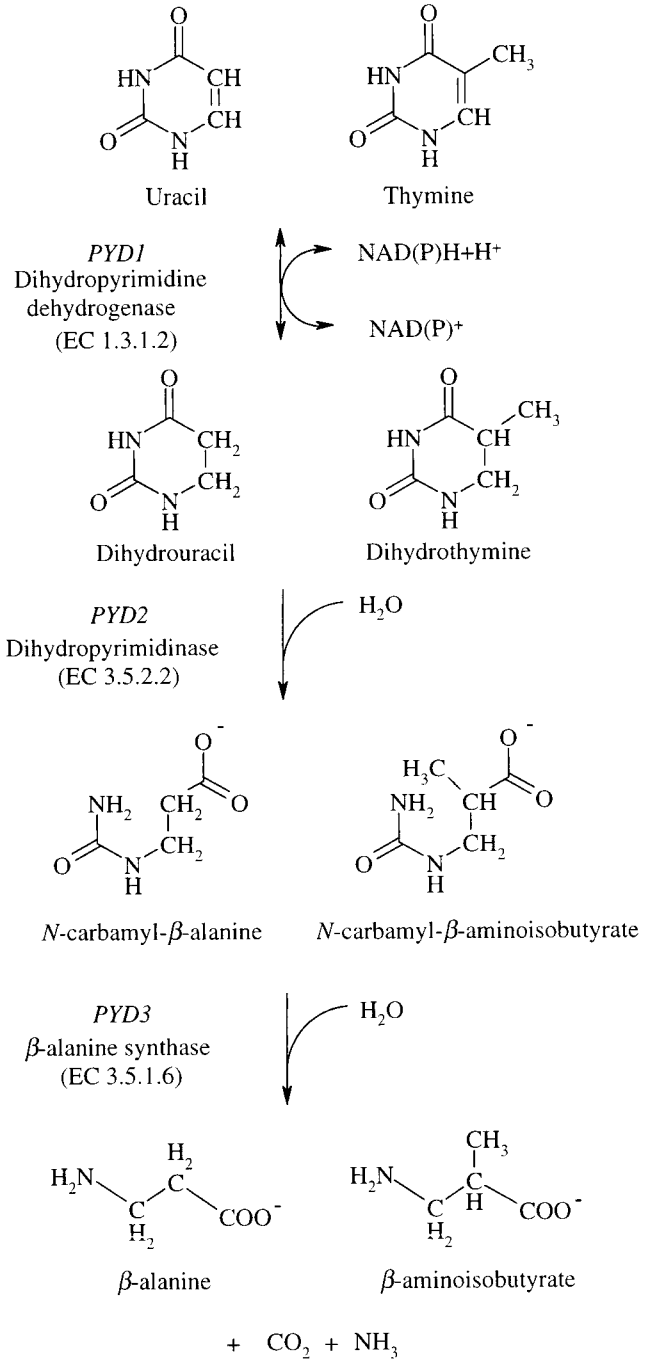



FIGURE 1.—Reductive catabolism of pyrimidines showing intermediates, enzymes, and the corresponding genes.

well-known neural inhibitor γ-amino-*n*-butyric acid (GABA), β-alanine is thought to function as a neurotransmitter (SANDBERG and JACOBSON 1981). Apart from having a well-described inhibitory function, GABA can also act as an excitatory transmitter (WAGNER *et al.* 1997), suggesting that β-alanine might also have the same effect. Disorders in the β-alanine metabolism of humans, such as hyper β-alaninemia, are associated with severe neural dysfunction, seizures, and death (HIGGINS *et al.* 1994). All clinical findings support the fact that well controlled β-alanine production is an important aspect of metabolic regulation (SCRIVER and GIBSON 1995). However, practically nothing is known about the regulation of β-alanine production at the genetic level in humans or any other organisms.

The majority of fungi, including *Saccharomyces cerevisiae*, cannot utilize pyrimidines or their degradation products as the sole source of nitrogen (LARUE and SPENCER 1968). Apparently, *S. cerevisiae* does not have the pyrimidine catabolic pathway. However, *S. kluyveri* has a functional degradation pathway and we developed a genetic system in this yeast to study the pyrimidine catabolic genes (GOJKOVIC *et al.* 1998, 2000). In this article we report on the *S. kluyveri PYD3* gene, encoding β-alanine synthase, and its regulation at the transcriptional level. In addition, we cloned and sequenced the analogous genes from *Dictyostelium discoideum* and *Drosophila melanogaster* and functionally expressed these in the *S. kluyveri pyd3-3* mutant. This work provides initial data on the origin of the pyrimidine catabolic pathway in eukaryotes.

## MATERIALS AND METHODS

**Strains and growth media:** The yeast strains used in this work are listed in Table 1. Yeast strains were grown at 25° in YPD medium (1% yeast extract, 2% bacto peptone, 2% glucose) or in defined minimal (SD) medium (1% succinic acid, 0.6% NaOH, 2% glucose, 0.67% yeast nitrogen base without amino acids; Difco, Detroit). When indicated, $(NH_4)_2SO_4$ was replaced either with 0.1% uracil, dihydrouracil, *N*-carbamyl-β-alanine, β-alanine, or proline as the sole nitrogen source. The growth rate was determined in liquid medium by following the optical density at 600 nm. The *Escherichia coli* strain DH5α was used for plasmid amplification. Bacteria were grown at 37° in Luria-Bertani medium supplemented with 100 mg/liter of ampicillin for selection (SAMBROOK *et al.* 1989). The *E. coli* BL21-CodonPlus (Stratagene, La Jolla, CA) strain was used for heterologous protein expression.

**Mutagenesis:** Yeast mutants were generated with ethyl methanesulfonate (EMS) using standard mutagenesis techniques (LAWRENCE 1991; GOJKOVIC *et al.* 1998). Mutagenized cells were plated on YPD medium, replicated on medium containing *N*-carbamyl-β-alanine as sole nitrogen source, and chosen by inability to grow on this medium. To test for recessiveness or dominance of the mutations, each mutant was crossed with a strain of the opposite mating type, either Y090 or Y091. Prototrophic diploid colonies were selected on SD medium and thereafter at least three colonies from each cross were tested for growth on *N*-carbamyl-β-alanine medium. Interallelic complementation tests were carried out by crossing Y1023 with Y1021 and Y1022.

**Cloning and DNA sequences analysis:** Transformation and complementation of *S. kluyveri* mutants was done by electroporation as described in GOJKOVIC *et al.* (2000) using the wild-type genomic library from *S. kluyveri.* This library, based on the Yep352 vector, was kindly provided by M. Costanzo (COSTANZO *et al.* 2000). *S. cerevisiae* was transformed by the lithium acetate method (GIETZ and SCHIESTL 1995). Plasmid DNA was purified from *E. coli* transformants on QIAGEN (Valencia, CA) columns. Both strands of the complete *PYD3* genes were sequenced using Thermo Sequenase radiolabeled terminator cycle sequencing kits (Amersham Pharmacia Biotech). Nucle-

**TABLE 1**

**The yeast strains used in this study**

| Strain | Genotype | Source |
|---|---|---|
| *S. kluyveri* Y057 (NRRL Y-12651) | Type strain (prototroph) | C. Kurtzman |
| *S. kluyveri* Y156 (GRY1175) | *MAT*α *ura3* | Weinstock and Strathern (1993) |
| *S. kluyveri* Y159 (GRY1183) | *MAT***a** *ura3* | Weinstock and Strathern (1993) |
| *S. kluyveri* Y090 | *MAT*α *thr* | L. Marsh |
| *S. kluyveri* Y091 | *MAT***a** *thr* aux$^-$ | L. Marsh |
| *S. kluyveri* Y1019 | *MAT*α *pyd2-1 ura3* | Gojkovic *et al.* (2000) |
| *S. kluyveri* Y1021 | *MAT*α *pyd3-1 ura3* | Derived from Y156 (this study) |
| *S. kluyveri* Y1022 | *MAT*α *pyd3-2 ura3* | Derived from Y156 (this study) |
| *S. kluyveri* Y1023 | *MAT***a** *pyd3-3 ura3* | Derived from Y159 (this study) |
| *S. cerevisiae* Y453 | *MAT***a** *ura3-52 ino1* | G. Fink |

Y, the laboratory yeast collection; aux$^-$, unidentified auxotrophic requirement.

otide sequence analysis and protein sequence comparisons were performed with Winseq (F. G. Hansen, unpublished software) and ClustalW 1.7 (Thompson *et al.* 1994) programs. Database searches were performed using the BLAST network services at the National Center for Biotechnology Information and the Saccharomyces Genome Database at Stanford Genomic Resources. The *S. kluyveri* 3361-bp nucleotide sequence, containing the *PYD3* gene, is listed in GenBank/EMBL databases under accession no. AF333185, the *D. discoideum PYD3* gene is under AF333186, and *D. melanogaster* is under AF333187.

**Plasmids:** The Yep352 library plasmid containing the full-length *S. kluyveri PYD3* gene is named P260. This plasmid was used for transforming *S. kluyveri pyd3* mutants and the *S. cerevisiae* Y453 strain. Expressed sequence tag (EST) clones from *D. discoideum* P399 (SSG647, GenBank accession no. C89941) and *D. melanogaster* P550 (GH 26887, GenBank accession no. AI513795), containing full-length cDNA, were obtained from the University of Tsukuba and Research Genetics (Birmingham, AL), respectively. For promoter studies the 918-bp *PYD3* promoter sequence, from −918 bp to the start codon, obtained by PCR using *Pfu* DNA polymerase (Stratagene), was inserted in the *Eco*RI/*Bam*HI sites of the plasmid pYLZ-2 (Hermann *et al.* 1992). The resulting plasmid was termed P289. A plasmid for heterologous expression in yeast, P403, was constructed by removing an *Age*I/*Hin*dIII part of the *GAL1* promoter from a pYES2 vector (Invitrogen, San Diego) and replacing it with a *PYD3* promoter. The β-alanine synthase cDNAs from *D. discoideum*, *D. melanogaster*, and human were cloned into P403, giving P492, P493, and P536, respectively. The C-terminal 8× His-tagged *E. coli* expression vector, P343, was constructed by inserting an *Eco*RI/*Hin*dIII fragment containing an eight-histidine tag from the plasmid pASKMh (Bader *et al.* 1998) and ligating it into the *Eco*RI/*Hin*dIII site of the pASK75. The *Xba*I and *Eco*RI sites of P343 were used to clone the *S. kluyveri PYD3* gene, giving a plasmid P491.

**DNA and RNA manipulation:** Yeast genomic DNA was isolated using zymolyase and standard procedures (Johnston 1994). Chromosomal DNA suitable for contour-clamped homogeneous electric field (CHEF) electrophoresis was obtained and separated according to Petersen *et al.* (1999). The VacuGene XL vacuum blotting system (Pharmacia Biotech, Piscataway, NJ) was used for blotting of chromosomes onto HYBOND-N+ nylon membrane (Amersham, Arlington Heights, IL). Total RNA was extracted from exponentially growing yeast cells using the FastRNA Red kit (BIO 101, Vista, CA) and a FastPrep machine FP 120 (BIO 101 Savant) according to the supplier. Poly(A) RNA was isolated with oligo(dT) cellulose (Sambrook *et al.* 1989). Total RNA (10 μg) applied in formaldehyde loading buffer was separated in a 1.2% agarose-formaldehyde gel run with MOPS buffer. RNA bands were capillary transferred to a HYBOND-N+ nylon membrane using 20× SSC. Hybridization with the random-primed $^{32}$P-labeled PCR fragment from P260 was carried out overnight. Membranes from Northern analyses were prehybridized at 65° (Sambrook *et al.* 1989) and hybridized at 42° in 50% formamide, 5× SSC, 2× Denhardt's reagent, 0.1% SDS, and 10% dextran sulfate. Membranes were washed twice with 2× SSC for 5 min at room temperature, twice with 2× SSC, 1% SDS for 30 min at 65°, and twice with 0.1× SSC for 20 min at 65°.

**Primer extension analysis:** Approximately 10 pmol of the synthetic oligonucleotide, PYD3PEXT: 5′-CTGGCGGAAACA GTAGTA-3′ was end labeled with [γ-$^{32}$P]ATP in a 20-μl reaction mixture with T4 polynucleotide kinase as described by the manufacturer (GIBCO BRL, Gaithersburg, MD). Two micrograms of poly(A)$^+$ RNA, isolated from cells grown on dihydrouracil medium, was incubated with 1 pmol end-labeled primer at 70° for 10 min and then placed on ice for 10 min. Four microliters of 5× first strand buffer, 2 μl of 0.1 m dithiothreitol, and 1 μl of 10 mm dNTPs were added to the reaction mixture. After incubation for 2–5 min at 37°, 200 units of SuperScript RNA H$^-$ reverse transcriptase (GIBCO BRL) was added and the mixture was incubated at 37° for 1 hr. The products were analyzed on 7% acrylamide sequencing gel.

**Enzyme assays and protein purification:** Yeast transformants were grown in an appropriate medium to an optical density of 1.0–1.5 at 600 nm. β-Galactosidase activity assays were performed after breaking cells with glass beads in a FastPrep machine. All values are expressed in Miller units (Miller 1972). The data presented were obtained from at least three independent transformants. Denaturing electrophoresis of the protein extracts was performed on SDS/PAGE (4.5% acrylamide stacking gel and 10% running gel) in the discontinuous buffer system (Laemmli 1970). Protein was quantified by the method of Bradford (1967). Bovine serum albumin served as a protein standard.

The β-alanine synthase activity was measured according to Van Kuilenburg *et al.* (1999). One unit is defined as the amount of enzyme that can catalyze the transformation of 1 μmol of *N*-carbamyl-β-alanine into β-alanine in 1 min at 37°. The [$^{14}$C]*N*-carbamyl-β-alanine was obtained from Moravek Biochemicals (Brea, CA). Cells were grown in 500 ml of either SD or proline/dihydrouracil medium until late exponential phase. The collected cells were suspended in 50 mm potassium phosphate (pH 7.5) and broken using a French press (1200

**TABLE 2**

**β-Alanine synthase activities in *S. kluyveri* Y159 (*PYD3*) and Y1023 (*pyd3-3*)**

| | β-Alanine synthase activity (milliunits/mg protein) | |
|---|---|---|
| Nitrogen source | Y159 | Y1023 |
| Ammonium sulfate | 2.54 | 3.42 |
| Proline + dihydrouracil | 56.37 | 0.84 |

β-Alanine synthase enzyme activity was measured in cells grown in media containing glucose as a carbon source and 0.5% ammonium sulfate or 0.1% proline and dihydrouracil as a nitrogen source supplemented with uracil to overcome auxotrophic requirements. The assays were performed at 37°. One unit is defined as the amount of enzyme that can catalyze the transformation of 1 μmol of substrate into product in 1 min under standard conditions.

p.s.i.). After centrifugation (13,000 × *g* for 15 min), proteins were assayed immediately in Tris buffer (50 mM Tris HCl, pH 7.5). The concentration of *N*-carbamyl-β-alanine was determined in a cell-free extract by a colorimetric procedure at 466 nm (West *et al.* 1982). The cells were grown to midexponential phase in 40 ml of appropriate medium, harvested by centrifugation, and crushed with glass beads in a FastPrep machine (40–60 sec at maximum speed). Samples were diluted with 0.1 M potassium phosphate buffer (pH 7.4) and cell-free extracts were obtained by centrifugation. For recombinant protein expression, *E. coli* cells were grown to a density of $A_{600\ nm}$ = 0.5–0.6 in Luria-Bertani medium supplemented with 100 μg/ml ampicillin. Protein expression was induced by 200 μg/liter of anhydrotetracycline hydrochloride (ACROS ORGANICS, NJ) for 10 hr at 25°. Collected cells were resuspended in buffer A (50 mM sodium phosphate, pH 8.0; 300 mM NaCl; 10% glycerol; 25 mM imidazole) and disrupted by French press (1000 p.s.i.). After centrifugation at 13,000 × *g* for 30 min, the supernatant was applied to a 5-ml $Ni^{2+}$-NTA column (QIAGEN). The column was washed with 10 volumes of buffer A, 10 volumes of buffer B (50 mM sodium phosphate, pH 6.0; 300 mM NaCl; 10% glycerol; 25 mM imidazole), and finally with 10 volumes of buffer B containing 50 mM imidazole. The recombinant β-alanine synthase was eluted from the column by a linear gradient of 50–500 mM imidazole in buffer B. Fractions containing recombinant protein were precipitated by ammonium sulfate (70% saturation at 0°), resuspended in Tris buffer (50 mM Tris HCl, pH 7.5; 100 mM NaCl; 1 mM DTT), and then applied to a G-25 column and stored at −80° at a concentration of 10 mg/ml.

## RESULTS

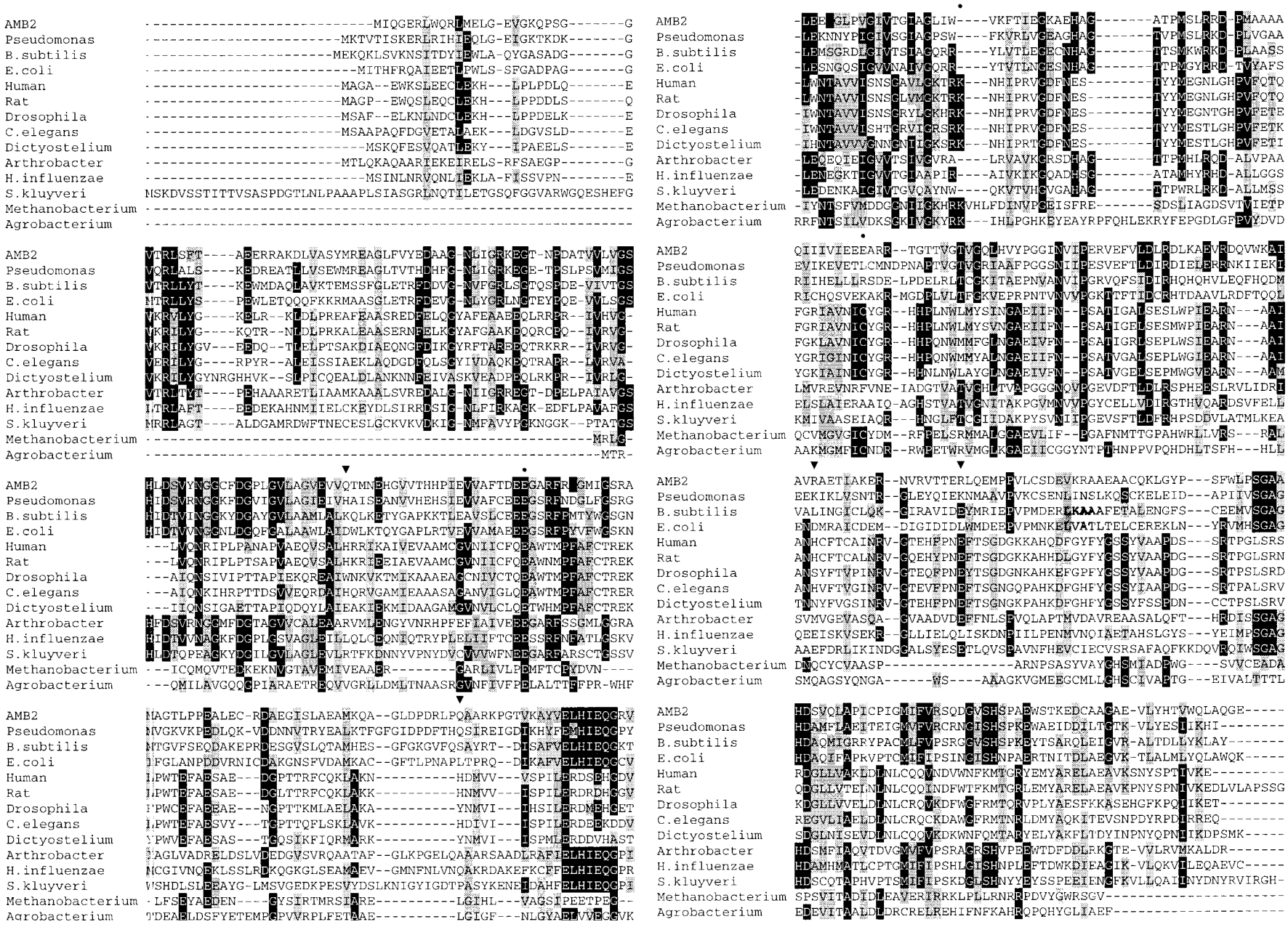
**Isolation of yeast mutants unable to grow on *N*-carbamyl-β-alanine as sole nitrogen source:** Several *S. kluyveri* mutants that are unable to grow on *N*-carbamyl-β-alanine as the sole nitrogen source were isolated after mutagenesis with EMS; they were named *pyd3* (*py*rimidine *d*egradation step three; Table 1). Besides their inability to grow on *N*-carbamyl-β-alanine, the mutants were not able to grow on uracil or dihydrouracil as sole nitrogen sources. Ammonium ions, which are necessary for growth, are liberated only in the last step of pyrimidine degradation. Detailed growth studies of the *pyd3* mutants revealed that they also could not grow on dihydrothymine (data not shown). However, growth of the *pyd3* mutants on β-alanine and β-aminoisobutyrate was not impaired, suggesting that the *pyd3* mutants probably bear a defect in the gene coding for β-alanine synthase. All *pyd3* mutations were recessive and fail to complement each other and thus are allelic.

No β-alanine synthase enzymatic activity was obtained from the *pyd3-3* mutant (Table 2). The *pyd3* mutant accumulates and excretes high amounts of the *N*-carbamyl-β-alanine (~6 μg/$10^8$ cells) when grown in the presence of 0.1% uracil, compared with 0.05 μg determined in the supernatants of proline + uracil-grown wild-type cells. These results suggest that the *pyd3* mutation is in the gene for the third catabolic activity or in a gene that regulates the third catabolic activity.

**Cloning and sequence analysis of the *PYD3* gene:** The *S. kluyveri pyd3 ura3* mutant strain, Y1023, was transformed to $Ura^+$ with an *S. kluyveri* genomic library. Several $Ura^+$ $Pyd^+$ colonies were identified. Colonies that lost the $Ura^+$ phenotype also lost the ability to grow on *N*-carbamyl-β-alanine, indicating that the complementing DNA was plasmid borne. Plasmids from these transformants were rescued into the *E. coli* DH5α strain, and all were identical. When this plasmid was introduced into the other two *pyd3* mutants, Y1021 and Y1022, it complemented their *pyd3* defect as well, supporting the idea that all three *pyd3* mutant genes are allelic. The plasmid contained a DNA insert with an open reading frame (ORF) of 1365 bp encoding a protein of 455 amino acids (Figure 2) with a $M_r$ of 49,707 and a pI of 5.2.

The deduced amino acid sequence encoded by the *S. kluyveri PYD3* gene exhibits similarity to carbamyl amino

Figure 2.—Multiple alignment of β-alanine synthases and related proteins. The sequences used for the comparison are the following: β-alanine synthases from human (NP057411), rat (Q03248), *Drosophila melanogaster* (AF333187), *Dictyostelium discoideum* (AF333186), *Caenorhabditis elegans* (U23139*), and *S. kluyveri* (AF333185); *N*-carbamyl-L-amino acid amidohydrolases from *Bacillus stearothermophilus* AMB2 (Q53389), *Pseudomonas* sp. (Q01264), *Bacillus subtilis* (Z99120*), *Escherichia coli* (P77425*), *Arthrobacter aurescens* (AF071221), and *Haemophilus influenzae* (Q57051*); and *N*-carbamyl-D-amino acid amidohydrolases from *Methanobacterium thermoautotrophicum* (AAB86277) and *Agrobacterium radiobacter* (AAB47607). The comparison was assembled with the ClustalW 1.7 program. Boxshade depicts all identical amino acids in white on black, similar amino acids are black on gray, while nonmatches are black on white. Putative proteins are marked by an asterisk (*). The residues involved in the active center of Agrobacterium *N*-carbamyl-D-amino acid amidohydrolases are marked by •. Ligands belonging to the two potential $Zn^{2+}$ binding sites that were determined for the rat enzyme are indicated by ▼.


AMB2
Pseudomonas
B.subtilis
E.coli
Human
Rat
Drosophila
C.elegans
Dictyostelium
Arthrobacter
H.influenzae
S.kluyveri
Methanobacterium
Agrobacterium

acid amidohydrolases from several bacteria. The highest similarity is to *Pseudomonas aeruginosa N*-carbamyl-β-alanine amidohydrolase, with an overall identity of 41% and a similarity of 56%. Similarity to other bacterial carbamyl amidohydrolases was in the range from 46 to 52%. While the bacterial enzymes catalyze a similar reaction, it is questionable whether they are directly involved in the degradation of pyrimidines. Mammalian β-alanine synthases show no significant similarity to the *S. kluyveri* enzyme. In general, only a few common structural features can be found among the aligned carbamyl amino acid amidohydrolases (Figure 2). Furthermore, comparison of *S. kluyveri* β-alanine synthase sequence to protein databases identified several candidates with an average sequence identity near 20% and an average similarity of 31–36%, including *N*-acyl-L-amino acid amidohydrolases (aminoacylases), indole-3-acetic amino acid hydrolases, carboxypeptidases, and aminotripeptidases from different organisms. *S. kluyveri* β-alanine synthase is longer than other eukaryotic carbamyl amidohydrolases due to the prolonged N terminus. (Figure 2). No Pyd3 homologues are encoded in the *S. cerevisiae* genome. The sequence upstream of the *PYD3* ORF has high similarity to the *S. cerevisiae* chitin synthase 3 gene (*CHS3*) located on chromosome II. The downstream sequence exhibits very high similarity to the chitin synthase 2 gene (*CHS2*), also on chromosome II, and to the chitin synthase 1 gene (*CHS1*) on chromosome XIV (data not shown). The region between the *S. cerevisiae CHS3* and *CHS2* genes spans 30 kb. However, in *S. kluyveri* the distance between these two genes is only 2.5 kb. Thus, it seems that this region is not very conserved among members of the Saccharomyces genus. After separation of the *S. kluyveri* chromosomes by CHEF electrophoresis and hybridization with the *PYD3* gene, we assigned *PYD3* to chromosome IV (data not shown).

The final proof that *PYD3* encodes an active pyrimidine catabolic enzyme was obtained by overexpression in *E. coli*. The *S. kluyveri PYD3* gene was subcloned into an *E. coli* expression vector, and the putative β-alanine synthase was expressed as a histidine-tagged protein (Figure 3). The purified enzyme could successfully convert *N*-carbamyl-β-alanine to β-alanine with the specific activity of 4.73 units/mg of protein. Thus, the *S. kluyveri PYD3* gene indeed codes for β-alanine synthase.

**Slime mold and fruit fly β-alanine synthase:** No potential *S. kluyveri PYD3* homologs were found in the *D. discoideum* and *D. melanogaster* EST databases. However, using the rat β-alanine synthase protein as a query, several EST homologs were identified. Sequencing of *D. discoideum* SSG647 clone revealed an ORF of 1176 bp encoding a protein of 391 amino acids (Figure 2) with a calculated molecular mass of 44 kD. Similarly, the *D. melanogaster* GH 26887 clone contained an 1161-bp ORF encoding a protein of 386 amino acids (Figure 2) with a predicted $M_r$ of 43,800. The GH 26887 sequence is in disagreement with a conceptual translation of the CG3027 gene product (GenBank accession no. AAF54141) published by Adams *et al.* (2000) as it is missing 60 bp close to the C terminus. Since the missing sequence contains 5′ and 3′ intron splicing sites and is not present in the cDNA clone, we consider it to be a third intron. In addition, when translated, the sequence within the 60-bp insert has no homology to any known β-alanine synthases. The *D. melanogaster PYD3* gene maps to region 84D11 of the Drosophila genome. So far, there are no known mutations that map to this region. The *D. discoideum PYD3* gene is similar to the mammalian β-alanine synthase (Figure 2). The gene is 54% identical to the rat β-alanine synthase and 53% identical to the human enzyme. The *PYD3* gene from *D. melanogaster* is ~63% identical to the human and rat enzyme with overall similarity of 77%. When compared to each other, the *D. discoideum* and *D. melanogaster PYD3* genes are 58% identical on the amino acid level. However, they do not show any close similarity to the *S. kluyveri PYD3* gene.

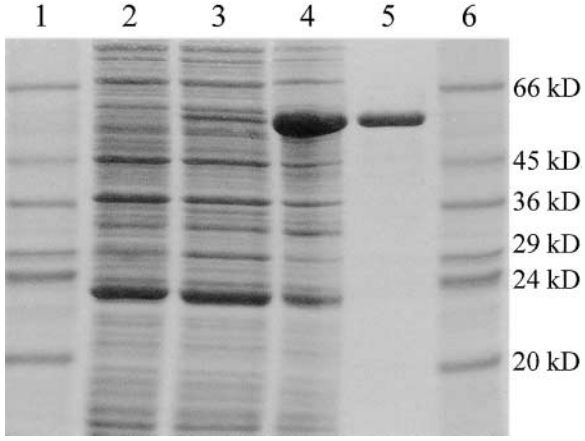



Figure 3.—SDS/PAGE of the recombinant *S. kluyveri* β-alanine synthase. The protein was expressed in *E. coli* BL21 CodonPlus and purified on a $Ni^{2+}$-NTA column. Standard proteins (lanes 1 and 6), homogenate of *E. coli* transformed with P343 (lane 2), proteins from noninduced *E. coli* transformed with P491 (lane 3), proteins from *E. coli* transformed with P491 at 10 hr after induction (lane 4), and concentrated purified heterologous *S. kluyveri* β-alanine synthase (lane 5) are shown. Proteins were visualized by Coomassie brilliant blue staining.

To demonstrate that the cloned genes are involved in pyrimidine catabolism, the cDNA sequences of the *D. discoideum* and *D. melanogaster PYD3* genes were placed under the control of the *S. kluyveri PYD3* promoter and introduced into Y1023. Both β-alanine synthase genes complemented the defect of the *S. kluyveri pyd3-3* mutant (Figure 4A). These results suggest strongly that *D. discoideum* and *D. melanogaster* genes code for a protein involved in pyrimidine catabolism. In addition, the β-alanine synthase gene from human (Vreken *et al.* 1999) also complemented the *S. kluyveri pyd3* mutation (Figure 4A).

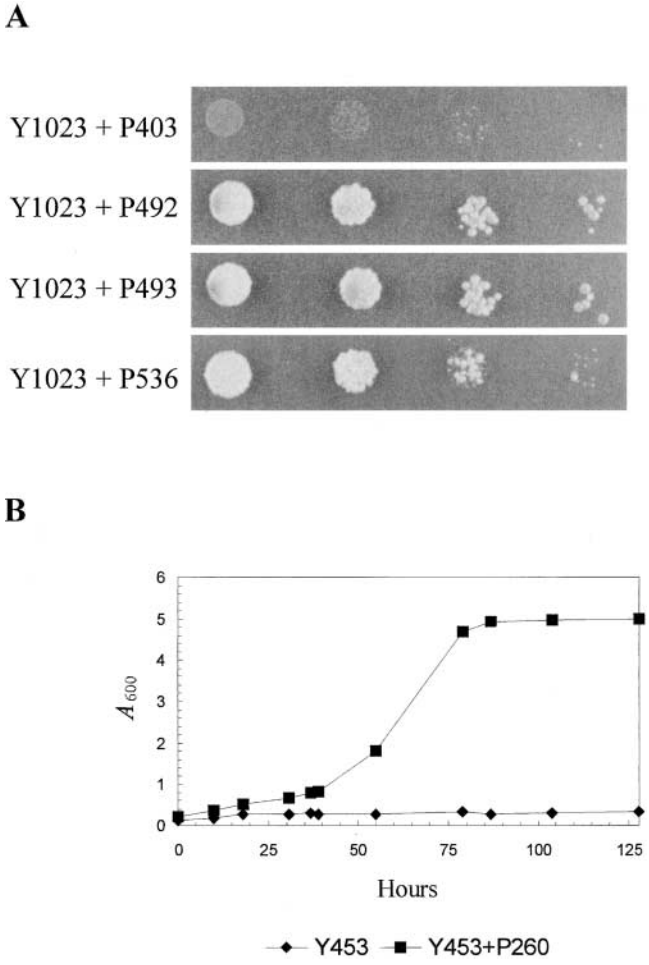



FIGURE 4.—Growth of *S. kluyveri* and *S. cerevisiae* containing heterologous β-alanine synthase genes (A). The Y1023 cells were transformed with P403, P492, P493, and P563, and the $Ura^+$ transformants were selected and grown in liquid SD medium overnight at 28°. The $A_{600}$ was adjusted to 0.1, and aliquots (5 μl) of serial 10-fold dilutions were spotted onto medium containing *N*-carbamyl-β-alanine as sole nitrogen source. The plates were photographed after incubation for 5 days at 25°. (B) Growth of the *S. cerevisiae* uracil-requiring strain Y453 transformed with the *S. kluyveri* β-alanine synthase gene. The Y453 cells were transformed with P260, selected for $Ura^+$ transformants on SD medium, inoculated into liquid media containing 0.1% *N*-carbamyl-β-alanine, and incubated at 28°. The growth rate was determined by following the absorbance at 600 nm. The medium for strain Y453 lacking the P260 plasmid was supplemented with uracil to overcome the *ura3* defect. (◆) Y453, (■) Y453 + P260.

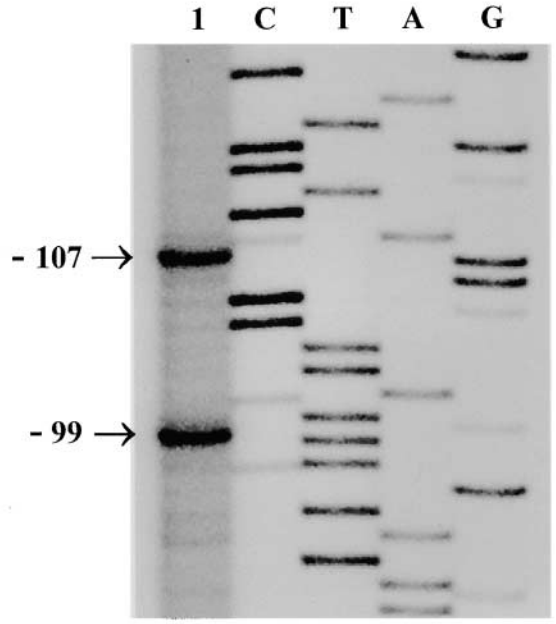
**The *PYD3* gene functions in *S. cerevisiae*:** *S. cerevisiae* cannot grow on *N*-carbamyl-β-alanine as sole nitrogen source (GOJKOVIC *et al.* 1998), which is not surprising since it does not have any genes encoding β-alanine synthase. Cells transformed with the *PYD3* gene grew on *N*-carbamyl-β-alanine and reached stationary phase after a few days, while nontransformed cells could not grow (Figure 4B).

**Analysis of yeast β-alanine synthase gene expression:** To localize the transcription initiation site of *S. kluyveri* β-alanine synthase mRNA, $Poly(A)^+$ RNA was isolated from the *S. kluyveri* type strain Y057 grown in dihydrouracil medium. Two transcription sites were detected at nucleotide positions −107 and −99 relative to the indicated AUG start codon (Figure 5A). The region upstream from the start codon (Figure 5B) was subcloned into a uracil-based high-copy plasmid, pYLZ-2, containing the *lacZ* gene as a reporter. The plasmid was transformed into *S. kluyveri* and independent transformants were grown in various media (Figure 5C). No β-galactosidase activity was observed when cells were grown in SD or on medium containing proline. High activity of the reporter gene was found in cells grown on dihydrouracil as the only source of nitrogen, while one-third of the activity, compared to that of dihydrouracil, was determined in the cells from SD + 0.1% dihydrouracil medium. It could be that ammonium ions inhibit transcription. The obtained results could also be explained that dihydrouracil transport is sensitive to nitrogen catabolite repression (NCR). In this case dihydrouracil would not be able to enter the cell in the presence of ammonium ions and subsequently induce the *PYD3* gene. Inability of ammonia to completely shut down the *PYD3* promoter in SD + dihydrouracil-grown cells may be attributed to a high-copy-number plasmid. Unfortunately, when the *PYD3* gene was present on a low copy plasmid no activity was detectable (data not shown).

The second reaction in the degradation of pyrimidines, hydrolysis of dihydrouracil to *N*-carbamyl-β-alanine, is activated by dihydrouracil (GOJKOVIC *et al.* 2000). Transcription of the *PYD3* gene is detectable only in cells grown in the presence of dihydrouracil or to a lesser extent with *N*-carbamyl-β-alanine as sole nitrogen sources (Figure 6). Using the yeast β-alanine synthase DNA as a probe against total *S. kluyveri* RNA, an mRNA band of ~1.5 kb was observed. If an alternative nitrogen source was present the mRNA was not detected (Figure 6). Thus transcription of *PYD3* mRNA is induced by dihydrouracil and *N*-carbamyl-β-alanine. However, because in the cell dihydrouracil is degraded by dihydropyrimidinase to *N*-carbamyl-β-alanine it is difficult to conclude if dihydrouracil indeed directly induces transcription of *PYD3*. To determine the time response of *PYD3* transcriptional activation, 0.1% dihydrouracil was added to cells grown on proline. Proline is considered to be a "neutral" nitrogen source in yeast and it does not interfere with regulation or uptake of other compounds (MAGASANIK 1992). No basal transcription of the *PYD3* gene was observed in cells grown on proline. However, 10 min after the addition of dihydrouracil it was possible to detect *PYD3* mRNA. After 60 min the same level was attained as in cells continuously grown on dihydrouracil (Figure 7, A and B).

Nitrogen catabolite repression is a physiological response by which, in the presence of a preferred nitrogen source, expression of the genes encoding catabolic en-

A




C

| Medium | β-galactosidase (Miller units) X 1000 | |
|---|---|---|
| | *PYD3p* | pYLZ-2 |
| SD | 0.0 | 0.01 |
| Dihydrouracil | 15.12 | 0.06 |
| SD + dihydrouracil | 4.0 | 0.02 |
| Proline | 0.01 | 0.0 |

B

```
                                                                    TGTCAAGTTTCGCGCTGA
-900  CGCTTCATCCAGGTCTTGCAAGGGAATGGCATCGTCCGGCGTGGCAGCTACGCCTGGGCCCGTCACAGAAGGGTCAACGCCGGTAGTAGA
-810  AGGTTGAACATTGATATGGTCCTTCTGCTCATTAGCCTTTTGAGCGTAGTAATAATGAGGATTCTGCGGATTGTTTAAATCGTTACGCTC
-720  CGGACGCACCAATGATCCCTGTCTGTGAACACCAATAGATTCGCTCCTAGAGCCACGAGCGACTCGCTATCAAAGCTCTGGTAGTCTTCG
-630  GACCTTCTGCTCTTTCTAGAAGACGAGTGCAGCATTGCTGTATCAATGGATATGGCTTGATGCGATGCGAAGAGGTAAGAGATCCTCGAA
-540  AGATCAAAATTTTTTTCTTCAAGTCCAGAAGAACGCAACTATCGAATGTTCCGTTTCAGGTTAAGTTCTATTTTTTCTCTTCGTTTTCGG
-450  TGCCCCAGAAACGCACGCGCGCATCATGATGCACGGAAAACTACTCCTCTCTGCCCACCTCCAGATTTGTTCATTCCCCAGCTCCTTTGA
-360  TCCGCTTCGGAAAAAAAAAAAAAAAAAAAAAAAAAAAACCGAAGGCTTTCGAATCGGAATTTATTAATGCATCGGTTACCGCGGCAGGGGT
-270  TCGAACACAGGCATTGTGATAATCTATGGTTGCAAACGCTATCTAACTGTATCAGTAACGATAGCATACACAGCTGATGATAACCGCCGG
                                                                    →     →
-180  AGTATGGCAACTCGTGAAAGGAGACCCTATACCTACCGTGCTTCGAGTTTACAACCAACGGTAGCATGCCTCAGGCCTTATTTGTATAAA
 -90  TAATCAGTGTTTTGTAGCGTTATATCTTGAGAAAGTTTTGGTCTTCTATCATCGATAATAAAAAATAAGCTATCCAAGAAATCATCAAAA
   1  ATG
      M
```

FIGURE 5.—Analysis of the *S. kluyveri PYD3* mRNA. (A) Mapping 5′ ends of *S. kluyveri N*-carbamyl-β-alanine amidohydrolase mRNAs. The 5′ ends of the 1.5-kb mRNAs were mapped with the primer PYD3PEXT in primer extension experiments using poly(A)$^+$ RNA from cells grown on dihydrouracil (lane 1). The signals corresponding to the transcription start points at positions −107 and −99 are marked by arrows. (B) The sequence of the promoter and 5′-untranslated mRNA of the *S. kluyveri PYD3* gene. The putative *cis* regulatory elements, $URS_{GATA}$, are boxed. An unusually long poly(A) sequence, located upstream from the start codon, is written in boldface type. (C) β-Galactosidase activity assays were performed on *S. kluyveri* Y156 transformed with the plasmid containing the *PYD3* promoter fused to the *lacZ* gene. The *PYD3* promoter sequence (*PYD3p*) containing 918 bp upstream of the start codon was cloned into the pYLZ-2 vector. pYLZ-2 denotes the high-copy plasmid without the *PYD3* promoter sequence.

zymes is severely decreased. Common *cis*-acting elements present in *S. cerevisiae* NCR-sensitive genes are GATA sequences (YOO and COOPER 1989; BYSANI *et al.* 1991). The *PYD3* upstream region contains several GATA-like sequences (Figure 5B). To assay whether the *S. kluyveri PYD3* catabolic gene is under NCR, we measured the level of *PYD3* transcription in the presence of a readily transported and metabolized nitrogen source,

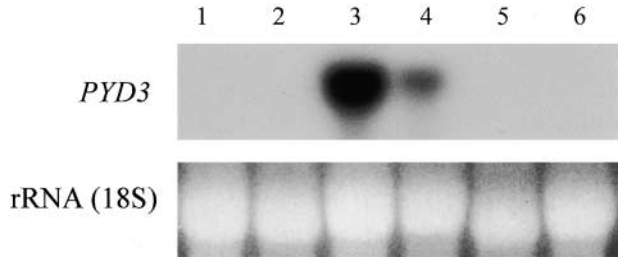



FIGURE 6.—Northern blot analysis of yeast *PYD3* expression. The prototrophic type strain cells of *S. kluyveri* Y057 were grown under various physiological conditions for several hours after which total RNA was isolated, blotted, and probed with a DNA fragment from the *PYD3* gene. The sole sources of nitrogen for growth of the cells are given for each lane. Lane 1, ammonia; lane 2, uracil; lane 3, dihydrouracil; lane 4, *N*-carbamyl-β-alanine; lane 5, β-alanine; and lane 6, proline. The small ribosomal subunit (18 S) was used as a control for equal loading.

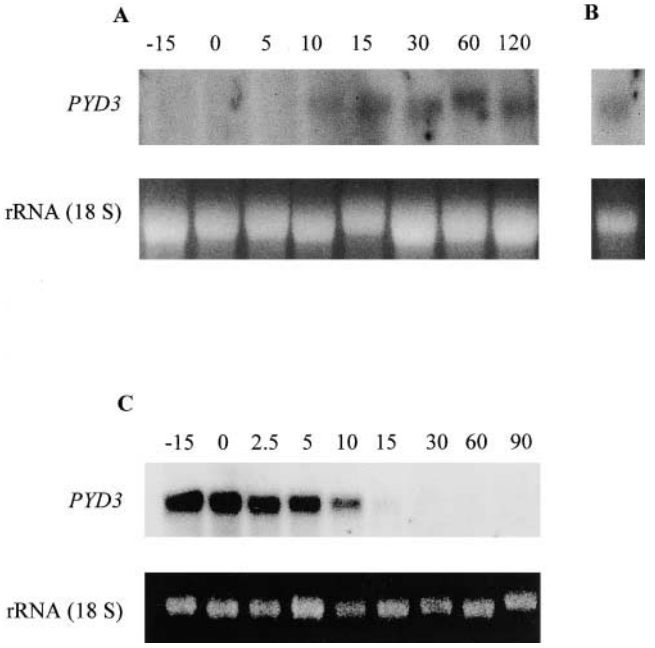



FIGURE 7.—Induction and nitrogen catabolite repression of *PYD3* transcription. (A) Induction of *PYD3* transcription by dihydrouracil shown as Northern analyses of cells grown in proline medium. A total of 40 ml of cells was collected at the indicated times (in minutes) and total RNA was isolated. A DNA fragment from the *PYD3* gene was used as a probe. Dihydrouracil (0.1%) was added at time point zero. (B) After ~30 min mRNA reached levels comparable to cells grown in dihydrouracil medium. (C) Nitrogen catabolite repression of transcription of the *PYD3* gene. Northern analysis of *PYD3* mRNA from cells grown in dihydrouracil medium and after the addition of 0.5% ammonia at 0 min. rRNA 18 S bands were used as loading controls. Ammonium ion represses completely the expression of the *PYD3* gene within 15 min after addition.

namely ammonia (Figure 7C). Shortly after the addition of ammonium sulfate, the level of *PYD3* mRNA began to decrease. *PYD3* transcription fell to undetectable levels between 10 and 15 min after the addition of this preferred nitrogen source (Figure 7C). The observed results could be explained that NCR works directly on expression of *PYD3* or, alternatively, only on the uptake of dihydrouracil. Anyhow, a regulatory pattern of *PYD3* expression resembles the one observed for the *S. kluyveri PYD2* gene (GOJKOVIC *et al.* 2000).

**The diversity and origin of β-alanine synthases:** There are remarkable similarities between the catabolic and the *de novo* biosynthetic pathways of pyrimidines. β-Alanine synthase catalyzes an almost reverse reaction of the second step of *de novo* pyrimidine biosynthesis. Therefore, it was expected that the yeast β-alanine synthase would exhibit similarity to the second enzyme in the *de novo* biosynthetic pathway, aspartate carbamyltransferase (ATCase). Only 14% sequence identity between *S. cerevisiae* ATCase and the *S. kluyveri* Pyd3 protein can be found, although KVALNES-KRICK and TRAUT (1993) reported 21.2% sequence identity between rat liver β-alanine synthase and *E. coli* ATCase. Substantially higher sequence similarity, almost 19%, was observed between the Pyd3 protein and ornithine carbamylase from *Schizosaccharomyces pombe*. Carbamyltransferases have a different catalytic mechanism from carbamyl amidohydrolases but bind very similar ligands. Multiple alignment and a phylogenetic analysis of available carbamyl amidohydrolases revealed that all the enzymes could be grouped into three subfamilies (Figure 8). The first subfamily includes bacterial *N*-carbamyl-L-amino acid amidohydrolases together with *S. kluyveri* enzyme and the putative huy-C protein from *Arabidopsis thaliana*. Among these, so far only the *S. kluyveri* enzyme is implicated in the catabolism of pyrimidines. The second subfamily consists of bacterial and Archaeal *N*-carbamyl-D-amino acid amidohydrolases, while the third subfamily includes mammalian and other eukaryotic putative β-alanine synthases. The tree topology did not change significantly when different calculation methods (maximum parsimony, maximum likelihood, clustering, or transformed distance) were used. The *S. kluyveri* enzyme, on both the nucleotide and amino acid levels, exhibits higher identity with bacterial than with mammalian carbamyl amidohydrolases. However, the Dictyostelium and Drosophila enzymes complemented a *pyd3* defect in *S. kluyveri*. As reported for mammals, the yeast enzyme is involved in the pyrimidine catabolic pathway and with β-alanine production, and, although not related structurally, the catalytic properties of the yeast and other eukaryotic enzymes must be similar.

## DISCUSSION

This article describes the cloning of the gene for β-alanine synthase by complementation of the *S. kluyveri pyd3* mutant as well as using this yeast system to characterize β-alanine synthases originating from *D. discoideum* and

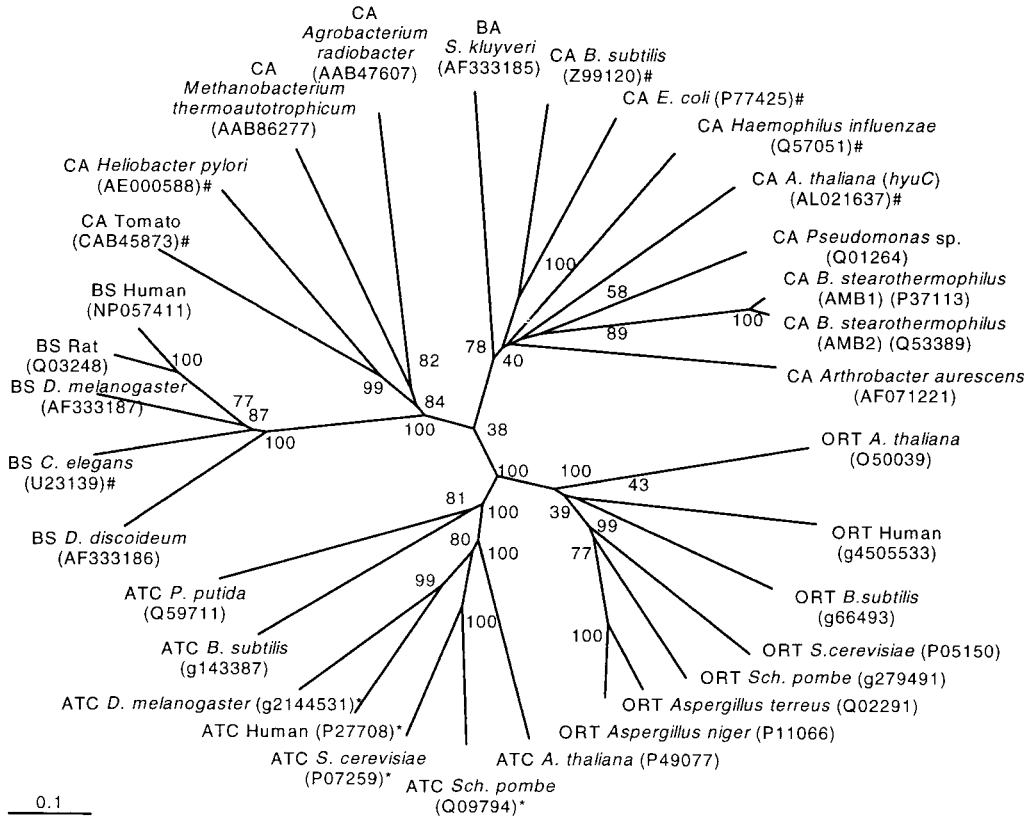



FIGURE 8.—A phylogenetic tree showing relations of carbamyl amidohydrolases with transcarbamylases. The phylogenetic tree was derived using the ClustalW 1.7 and TreeView 1.5.2 programs (http://taxonomy.zoology.gla.ac.uk/rod/fod.html). The numbers given on the branches are frequencies (written as percentages) at which a given branch appeared in 1000 bootstrap replications. The enzyme names are as follows: CA, carbamyl amidohydrolase; BS, β-alanine synthase; ATC, aspartate transcarbamylase (EC 2.1.3.2); ORT, ornithine carbamoyltransferase (EC 2.1.3.3). Putative homologous enzymes based on sequence similarities are marked #. Only the ATCase domain of multifunctional enzymes was used for comparison (*). Accession numbers are in parentheses.

*D. melanogaster*. The *S. kluyveri* β-alanine synthase is closely related to bacterial *N*-carbamyl-L-amino acid amidohydrolases. However, the role of bacterial *N*-carbamyl amidohydrolases in pyrimidine catabolism has never been elucidated. Surprisingly, amino acid similarity between yeast and other eukaryotic putative β-alanine synthases is not very evident. Apparently only a few structural features are common for all these carbamyl amidohydrolases. One of the conserved residues shared by all the amidohydrolases that were compared is a glutamic acid toward the N termini of the proteins. A less conserved, very hydrophobic region precedes this invariant residue. The same glutamic acid residue is a part of a conserved motif shared by rat liver *N*-carbamyl-β-alanine amidohydrolase, nitrilases, cyanide hydratases, and aliphatic amidases (BORK and KOONIN 1994). All of these proteins constitute a family of carbon-nitrogen hydrolase enzymes, and it was suggested that this residue might be involved in catalysis (BORK and KOONIN 1994). Indeed, the recent report of the crystal structure of *Agrobacterium sp. N*-carbamyl-D-amino acid amidohydrolase confirmed involvement of Glu46 in the active center (NAKAI *et al.* 2000). Lys126, also involved in the active center, is present in *D. discoideum* and *D. melanogaster* β-alanine synthase but not in the *S. kluyveri* enzyme. Another highly conserved motif, which contains an invariant cysteine, was shown to be a part not only of the Agrobacterium amidohydrolase (GRIFANTINI *et al.* 1996; NAKAI *et al.* 2000) but also of the active center of nitrilases (KOBAYASHI *et al.* 1992). However, this residue is not present in the *S. kluyveri* enzyme, which has isoleucine at this position, but it is conserved in both the *D. discoideum* and *D. melanogaster* enzymes. It is clear that all *N*-carbamyl-D-amino acid amidohydrolases and eukaryotic β-alanine synthases, except one from *S. kluyveri*, have a conserved catalytic center (Glu-Lys-Cys) but otherwise differ in overall structure. Agrobacterium amidohydrolase is much shorter compared to the mammalian enzymes and it forms a homotetramer while the rat β-alanine synthase exists as a homohexamer. Until now, only the rat and the human enzymes proved to be directly involved in the catabolic pathway. In contrast, many bacterial carbamyl amidohydrolases cannot use *N*-carbamyl-β-alanine as substrate (OGAWA *et al.* 1994; NANBA *et al.* 1998; WILMS *et al.* 1999), and they may not participate in the degradation of pyrimidines. The sequence similarity among carbamyl amidohydrolases is not very high; in a phylogenetic tree they group together but are divided into three subfamilies. Identical results were consistently obtained, despite using various calculation methods, which supports the idea of a single ancestral gene.

The pyrimidine catabolic pathway shows similarity to *de novo* pyrimidine biosynthesis. For example, a cysteine, observed in the active center of the dihydroorotate dehydrogenase (fourth step in the *de novo* pathway; BJÖRNBERG *et al.* 1997; ROWLAND *et al.* 1998), is also present in the first catabolic enzyme: dihydrouracil dehydroge-

nase. Similarity between the second catabolic enzyme in yeast, dihydropyrimidinase, and dihydroorotases, which catalyzes the third step of *de novo* biosynthesis, was also observed (Gojkovic *et al.* 2000). The third catabolic enzyme exhibits only a limited degree of homology to the enzyme catalyzing the second *de novo* biosynthetic reaction. The similarity between β-alanine synthase and the second enzyme in the *de novo* biosynthetic pathway, ATCase, is only 20%. In addition to aspartate and ornithine transcarbamylase (Kvalnes-Krick and Traut 1993), the rat β-alanine synthase shows sequence similarities with several diverse proteins involved in the reduction of organic nitrogen compounds (Bork and Koonin 1994). These observations, together with a proposed molecular evolution of carbamyltransferases (Labedan *et al.* 1999), suggest that the last common ancestor to all existing life already had differentiated copies of genes coding for transcarbamylases and carbamyl amidohydrolases. Following differentiation, the carbamyl amidohydrolases with a broad substrate specificity for different carbamyl amino acids evolved into pyrimidine catabolic proteins and "specialized" in the degradation of *N*-carbamyl-β-alanine. However, to understand fully the ancestral pattern of these enzymes it is necessary to answer also the following question: What is the biological function of different L- and D-carbamyl amino acids and carbamyl amidohydrolase activity in the cell? It could be that active carbamyl groups, for example, carbamyl phosphate, attach to several amino acids in the cell, generating carbamyl amino acids. These compounds are then detoxified with carbamyl amidohydrolases. On the other hand, in some organisms carbamyl amidohydrolases could be necessary for generation of D-amino acids, which are constituents of toxins in frog skin, for instance (Birk *et al.* 1989; Kreil 1997).

*S. cerevisiae* does not have a β-alanine synthase gene and cannot degrade *N*-carbamyl-β-alanine. However, when transformed with the *S. kluyveri PYD3* gene, *S. cerevisiae* gains the ability to utilize *N*-carbamyl-β-alanine as a sole nitrogen source. It is clear, therefore, that *S. cerevisiae* can transport *N*-carbamyl-β-alanine, although this compound cannot be degraded by wild-type *S. cerevisiae*. When a reporter gene linked to the *PYD3* promoter was introduced into *S. cerevisiae*, no activity resulted (data not shown). Apparently *S. cerevisiae* lacks not only the structural genes necessary for pyrimidine catabolism, but also some of the regulatory elements responsible for full induction of this pathway. However, a low basal level of *PYD3* gene transcription must occur, judging from the growth of transformed *S. cerevisiae* on *N*-carbamyl-β-alanine, despite the fact that we did not detect β-galactosidase activity when the *PYD3* promoter preceded the gene.

The expression of the β-alanine synthase gene in *S. kluyveri* is regulated at the level of transcription. The transcription of the *PYD3* gene is induced in the presence of dihydrouracil and *N*-carbamyl-β-alanine. A similar situation was observed for the *PYD2* gene, which encodes the second pyrimidine catabolic enzyme, 5,6-dihydropyrimidine amidohydrolase (Gojkovic *et al.* 2000). Contrary to the situation described for mammals and some bacteria, uracil does not act as an activator of this pathway in yeast. *PYD3* expression is undetectable when ammonia is present, even in the presence of an inducer. This observation leads us to hypothesize that NCR is superimposed over induction of *PYD3* or that dihydrouracil transport is sensitive to NCR and thus the inducer cannot be transported into the cell. So far, we have not been able to determine the *cis*-acting sequences responsible for dihydrouracil/*N*-carbamyl-β-alanine induction, but preliminary results suggest that there are several such sequences present in the minimal promoter. The *PYD3* gene may be under the same regulation as the *S. kluyveri PYD2* gene (Gojkovic *et al.* 2000), and it is apparent that the promoters from both genes possess common short motifs, but so far it is not clear if these represent the pathway-specific *cis*-acting elements. On the other hand, it is likely that the *PYD1* gene encoding dihydropyrimidine dehydrogenase is under different regulation, especially regarding the role of dihydrouracil and *N*-carbamyl-β-alanine.

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