Physiological Characterization of the *ARO10*-Dependent, Broad-Substrate-Specificity 2-Oxo Acid Decarboxylase Activity of *Saccharomyces cerevisiae*

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Aerobic, glucose-limited chemostat cultures of *Saccharomyces cerevisiae* CEN.PK113-7D were grown with different nitrogen sources. Cultures grown with phenylalanine, leucine, or methionine as a nitrogen source contained high levels of the corresponding fusel alcohols and organic acids, indicating activity of the Ehrlich pathway. Also, fusel alcohols derived from the other two amino acids were detected in the supernatant, suggesting the involvement of a common enzyme activity. Transcript level analysis revealed that among the five thiamine-pyrophospate-dependent decarboxylases (*PDC1*, *PDC5*, *PDC6*, *ARO10*, and *THI3*), only *ARO10* was transcriptionally up-regulated when phenylalanine, leucine, or methionine was used as a nitrogen source compared to growth on ammonia, proline, and asparagine. Moreover, 2-oxo acid decarboxylase activity measured in cell extract from CEN.PK113-7D grown with phenylalanine, methionine, or leucine displayed similar broad-substrate 2-oxo acid decarboxylase activity. Constitutive expression of *ARO10* in ethanol-limited chemostat cultures in a strain lacking the five thiamine-pyrophosphate-dependent decarboxylases, grown with ammonia as a nitrogen source, led to a measurable decarboxylase activity with phenylalanine-, leucine-, and methionine-derived 2-oxo acids. Moreover, even with ammonia as the nitrogen source, these cultures produced significant amounts of the corresponding fusel alcohols. Nonetheless, the constitutive expression of *ARO10* in an isogenic wild-type strain grown in a glucose-limited chemostat with ammonia did not lead to any 2-oxo acid decarboxylase activity. Furthermore, even when *ARO10* was constitutively expressed, growth with phenylalanine as the nitrogen source led to increased decarboxylase activities in cell extracts. The results reported here indicate the involvement of posttranscriptional regulation and/or a second protein in the *ARO10*-dependent, broad-substrate-specificity decarboxylase activity.

Saccharomyces cerevisiae has a narrow range of carbon sources that support growth (1) but is considerably more flexible with respect to the utilization of nitrogen sources (2). Most amino acids can be utilized as sole nitrogen sources but not as sole carbon sources for growth (28). The most common mechanism for utilizing amino acids as nitrogen sources is transamination, using 2-oxoglutarate or other 2-oxo acids as amino acceptors. This process leaves the carbon skeleton of the amino acid intact, in the form of a 2-oxo acid. For some amino acids (e.g., alanine), the resulting 2-oxo acid, pyruvate, can be readily cometabolized in central metabolism. In other cases, such as for the aromatic and branched-chain amino acids, the 2-oxo acids resulting from transamination are not intermediates of central metabolism. Even though they cannot be used as auxiliary carbon sources, these compounds are often trans-formed by the yeast cells before they are excreted into the growth medium.

An important and common pathway for catabolism of amino acids by yeasts is called the Ehrlich pathway (7–12, 37). This pathway is initiated by transamination of the amino acid to the corresponding 2-oxo acid. This 2-oxo acid is then decarboxylated to the corresponding aldehyde. Depending on the redox status of the cells (44), the aldehydes can then be reduced by alcohol dehydrogenases (yielding a group of compounds commonly referred to as fusel alcohols) or be oxidized to the corresponding organic acid ("fusel acids") by aldehyde dehydrogenases (Fig. 1). The fusel alcohols and their esters are especially important contributors to the flavor and aroma of fermented beverages (6, 16, 45). Phenylethanol, which has a typical rose-like flavor, can be produced by biotransformation of phenylalanine with *S. cerevisiae* cell suspensions (38, 39).

The identity of the decarboxylase(s) that catalyzes the initial step of the Ehrlich pathway has recently been investigated in our laboratories (7, 9–11, 44). The *S. cerevisiae* genome contains five genes that share sequence similarities with genes encoding thiamine pyrophosphate (TPP)-dependent decar-

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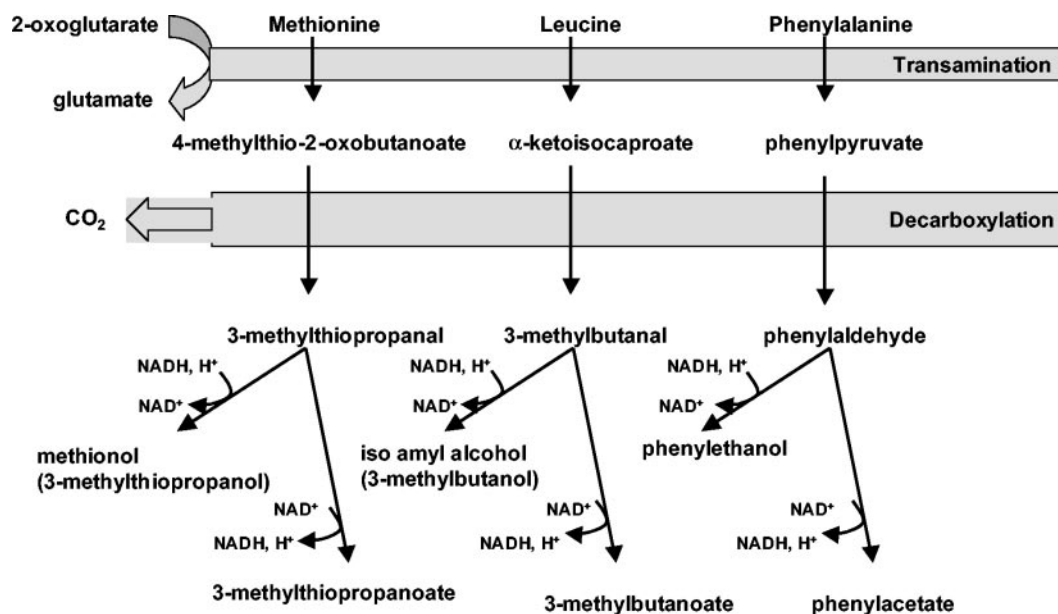
FIG. 1. Formation of fusel alcohols and fusel organic acids during the catabolism of the amino acids leucine, phenylalanine, and methionine.

boxylases (19, 20, 27) (for a review, see reference 21). Three of these genes (*PDC1*, *PDC5*, and *PDC6*) encode pyruvate decarboxylases. *PDC1* and *PDC5* encode the major pyruvate decarboxylases under most cultivation conditions (15, 20); *PDC6* is specifically expressed under low-sulfur conditions and encodes a pyruvate decarboxylase that has a low content of sulfur-containing amino acids (4, 14). Mutants in which all three *PDC* genes have been inactivated, and which completely lack pyruvate decarboxylase activity, still express branched-chain and aromatic 2-oxo acid decarboxylase activities (7, 40, 44). The other two members of this gene family are *ARO10* and *THI3*. Based on studies with deletion mutants, both have been implicated in the decarboxylation of branched-chain and aromatic 2-oxo acids (7, 10, 44). In addition, Thi3p has been assumed to be a positive regulator of the thiamine biosynthetic pathway. Upon its deletion, the transcription of all the genes of thiamine biosynthesis was negatively affected (13, 21). An *aro10 thi3* double-deletion mutant completely lacks phenylpyruvate decarboxylase activity, whereas the single-deletion mutants in these genes retain this enzyme activity (44). This might lead to the simple conclusion that both genes encode active phenylpyruvate decarboxylases. However, the situation is more complicated, as *pdc1 pdc5 pdc6 thi3* quadruple-deletion mutants, but not *pdc1 pdc5 pdc6 aro10* mutants, express phenylpyruvate decarboxylase activity (7, 10, 44). These and other observations have led to the proposal that *THI3* may not by itself encode an active phenylpyruvate decarboxylase but requires the simultaneous expression of one of the *PDC* genes to contribute to phenylpyruvate decarboxylase activity (44). This provided a first indication that the regulation and substrate specificities of the TPP-dependent decarboxylases in *S. cerevisiae* may be more complicated than a simple situation in which substrate specificity is determined by a mixture of five decarboxylases with defined—if overlapping—substrate specificities and kinetics.

With the exception of the transcriptional regulation of *ARO10* by aromatic amino acids modulated by the positive transcription factor *ARO80* (24), comparatively little is known about the regulation of fusel alcohol production in *S. cerevisiae* and the impact of the expression levels of the decarboxylase genes on the rates of production of the different decarboxylases.

The aim of the present study was to analyze the substrate specificity of the *ARO10*-dependent decarboxylase activity in *S. cerevisiae*, its impact on the production of fusel alcohols and acids, and the importance of transcriptional regulation in controlling its in vivo activity. To this end, we correlated the expression of *ARO10* (as well as that of the other decarboxylase genes) with the levels of fusel alcohols and acids in chemostat cultures of *S. cerevisiae* grown with different nitrogen sources. Subsequently, we investigated the substrate specificity of the *ARO10*-dependent decarboxylase activity and the impact of transcriptional regulation of *ARO10* on this activity by constitutively expressing *ARO10* in a wild-type *S. cerevisiae* strain, as well as in a *pdc1 pdc5 pdc6 aro10 thi3* quintuple-null mutant.

MATERIALS AND METHODS

Strains. The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1.

Recombinant-DNA techniques. Standard protocols were followed for plasmid isolation, restriction, ligation, transformation, and gel electrophoresis (30). Yeast chromosomal DNA was isolated by a method described previously (22). *S. cerevisiae* strains were transformed using the lithium acetate–single-stranded carrier DNA–polyethylene glycol method (17).

Overexpression of ARO10. The *ARO10* (YDR380W) open reading frame was PCR amplified from CEN.PK113-7D genomic DNA using primers *ARO10*-fwd (GGTCTAGAATGGCACCTGTTACAATTGAAAAG) and *ARO10*-rev (GGCTCGAGCTATTTTTTATTTCTTTTAAGTGCCGC), designed to introduce restriction sites (underlined) for endonuclease XbaI upstream of the ATG and XhoI downstream of the stop codon, respectively. The PCR product and the vector p426GPD (31) were digested by XbaI and XhoI. The XbaI-XhoI PCR fragment was directionally cloned behind the glyceraldehyde-3-phosphate dehy-

TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source or reference
CEN-PK 113-7D	*MAT**a** MAL2-8c SUC2* isogenic prototrophic strain	P. Kötter[a]
CEN-PK 113-5D	*MAT**a** MAL2-8c SUC2 ura3*	P. Kötter
CEN-PK 555-4D	*MAT**a** MAL2-8c SUC2 aro10Δ*	44
CEN-PK 711-7C	*MAT**a** MAL2-8c SUC2 ura3 pdc1Δ pdc5Δ pcd6Δ aro10Δ thi3Δ*	This study
IMZ001	*MAT**a** MAL2-8c SUC2 ura3 pdc1Δ pdc5Δ pcd6Δ aro10Δ thi3Δ* p426GPD (*URA3*)	This study
IMZ002	*MAT**a** MAL2-8c SUC2 ura3 pdc1Δ pdc5Δ pcd6Δ aro10Δ thi3Δ* pUDe001 (*URA3 TDH3p-ARO10*)	This study
IME003	*MAT**a** MAL2-8c SUC2 ura3* pUDe001 (*URA3 TDH3p-ARO10*)	This study
IME004	*MAT**a** MAL2-8c SUC2 ura3* p426GPD (*URA3*)	This study

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drogenase promoter (*TDH3p*) into p426GPD, resulting in plasmid pUDe001. The *ARO10* open reading frame sequence was confirmed by sequencing. The plasmid pUDe001 was transformed by the lithium acetate–single-stranded carrier DNA–polyethylene glycol method (17) into the *S. cerevisiae* CEN.PK 113-5D strain, resulting in strain IME003, and into strain CEN.PK 711-7C, resulting in strain IMZ002. Similarly, CEN-PK 113-5D and CEN.PK 711-7C were transformed with p426GPD (31), resulting in IME002 and IMZ001, respectively.

Chemostat cultivation. Aerobic chemostat cultivation was performed at 30°C in 1-liter working volume laboratory fermentors (Applikon, Schiedam, The Netherlands) at a stirrer speed of 800 rpm, pH 5.0, with a dilution rate (D) of 0.10 h^{-1} as described previously (42), with the exception of the strains IMZ001 and IMZ002, which were grown at a dilution rate of 0.05 h^{-1}. The pH was kept constant, using an ADI 1030 biocontroller (Applikon, Schiedam, The Netherlands), via the automatic addition of 2 M KOH. The fermentor was flushed with air at a flow rate of 0.5 liter min^{-1} using a Brooks 5876 mass-flow controller (Brooks Instruments, Veenendaal, The Netherlands). The dissolved-oxygen concentration was continuously monitored with an Ingold model 34 100 3002 probe (Mettler-Toledo, Greifensee, Switzerland) and was above 50% of air saturation.

Carbon-limited steady-state chemostat cultures of *S. cerevisiae* strains were grown as described previously (43) on synthetic medium containing 7.5 g of glucose liter^{-1} or 5.7 g liter^{-1} of ethanol, keeping molar carbon equivalence constant at 0.25 M, and either 5.0 g liter^{-1} (NH$_4$)$_2$SO$_4$, 5.0 g liter^{-1} of L-phenylalanine (44), 10 g liter^{-1} L-leucine, 11.3 g liter^{-1} L-methionine, 5 g liter^{-1} L-asparagine, or 8.8 g liter^{-1} L-proline as the sole nitrogen source. The absence of (NH$_4$)$_2$SO$_4$ was compensated for by the addition of equimolar amounts of K$_2$SO$_4$ when phenylalanine, leucine, methionine, proline, or asparagine was used as the only nitrogen source.

Culture dry weight. Culture dry weights were determined via filtration as described previously (35).

Extracellular-metabolite analysis. For the determination of phenylalanine, leucine, and methionine catabolism products and carbon recovery, culture supernatants and media were analyzed by high-performance liquid chromatography (HPLC), fitted with an AMINEX HPX-87H ion-exchange column (300 by 7.8 mm; Bio-Rad) mounted in a Waters Alliance 2690 HPLC apparatus, at 60°C using H$_2$SO$_4$ as the mobile phase with a flow rate of 0.6 ml · min^{-1}. Metabolites were detected by a dual-wavelength absorbance detector (Waters 2487) and a refractive-index detector (Waters 2410) and integrated with Chrompack Maitre 2.5 software.

Identification of metabolites by NMR spectroscopy. After lyophilization, samples of culture supernatants were dissolved in D$_2$O. ^{1}H, ^{1}H-^{1}H TOCSY, and ^{1}H-^{13}C correlation spectra (direct and long range) were measured at 300 K on a Bruker Avance 600 nuclear magnetic resonance (NMR) spectrometer equipped with an inverse triple-resonance probe and a pulse field gradient system. Quantitative ^{1}H-NMR experiments were also performed at 600 MHz. To 0.5 ml of supernatant, an equal amount of a standard solution containing maleic acid and EDTA was added. After lyophilization, the residue was dissolved in D$_2$O and the ^{1}H-NMR spectrum was measured using a relaxation delay of 30 seconds, ensuring full relaxation of all the hydrogen atoms between pulses. The integrals of the characteristic resonances for each component and the internal standard (singlet at 6.1 ppm) were measured, and the contents of the individual components were calculated.

Preparation of cell extracts. For the preparation of cell extracts, culture samples were harvested by centrifugation; washed twice with 10 mM potassium-phosphate buffer, pH 7.5, containing 2 mM EDTA; concentrated fourfold; and stored at −20°C. Before cell breakage, the samples were thawed at room temperature, washed, and resuspended in 100 mM potassium phosphate buffer, pH 7.5, containing 2 mM MgCl$_2$ and 2 mM dithiothreitol. Extracts were prepared by

sonication with 0.7 mm glass beads at 0°C for 2 min at 0.5-min intervals with an MSE sonicator (150-W output; 8-μm peak-to-peak amplitude). Unbroken cells and debris were removed by centrifugation at 4°C (20 min; 36,000 × g). The purified cell extract was used for enzyme assays.

2-Oxo acid decarboxylase assays. 2-Oxo acid decarboxylase activity was measured at 30°C immediately after preparation of cell extracts using a coupled reaction. Activity was measured by following the reduction of NAD$^+$ at 340 nm in the presence of excess aldehyde dehydrogenase from yeast. The reaction mixtures contained, in a total volume of 1 ml, 100 mM KH$_2$PO$_4$/K$_2$HPO$_4$ buffer, pH 7.0; 2 mM NAD$^+$; 5 mM MgCl$_2$; 15 mM pyrazole; 0.2 mM thiamine diphosphate; 1.75 U of yeast aldehyde dehydrogenase (Sigma-Aldrich, Zwijndrecht, The Netherlands) (dissolved in 1 mM dithiothreitol); and 2 mM phenylpyruvic acid, α-ketoisocaproate, α-ketoisovalerate, α-ketomethylvalerate, 3-methylthio-α-ketobutyrate, or pyruvate to initiate the reaction. Reaction rates were linearly proportional to the amount of cell extract added.

Activity data normalization. The per-strain normalization accounts for the difference in detection efficiency between 2-oxo acid decarboxylase activities. It also allows comparison of the relative change in activity levels, as well as displaying these levels in similar scales on the same graph. GeneSpring (Silicon Genetics, Redwood City, CA) uses the following formula to normalize to the median for each strain: (activity of strain X on substrate Y)/(median of every measurement of strain X).

Protein determination. Protein concentrations in cell extracts were determined by the Lowry method (29). Bovine serum albumin (fatty acid free; Sigma, St. Louis, Mo.) was used as a standard.

Microarray analysis. DNA microarray analyses were performed with the S98 Yeast GeneChip arrays from Affymetrix as previously described (34). Cells were transferred directly from chemostats into liquid nitrogen and processed according to the manufacturer's instructions (Affymetrix technical manual; Affymetrix, Santa Clara, CA.). Data analyses were performed with the Affymetrix software packages Microarray Suite v5.0, MicroDB v3.0, and Data Mining Tool v3.0. The Significance Analysis of Microarrays (SAM version 1.12) (41) add-in to Microsoft Excel was used for comparisons of replicate array experiments.

RESULTS

Measurement of phenylalanine and phenylethanol in chemostat culture of *S. cerevisiae* grown on various nitrogen sources. The *S. cerevisiae* reference strain CEN.PK113-7D was grown on synthetic medium in aerobic, glucose-limited chemostat cultures with different nitrogen sources: ammonium sulfate, phenylalanine, leucine, methionine, proline, or asparagine. During growth on phenylalanine as the nitrogen source, HPLC analysis of culture supernatants revealed the presence of high concentrations of phenylethanol and phenylacetate, consistent with the operation of the Ehrlich pathway. Surprisingly, low but significant concentrations of these metabolites were also observed when leucine or methionine was the sole nitrogen source (Table 2). The concentrations of phenylethanol and phenylacetate in leucine- and methionine-grown cultures were 20- to 50-fold higher than in cultures grown with ammonium sulfate as the nitrogen source (Table 2). Similarly,

2-methylpropanoate and *p*-hydroxyphenylacetate, which are Ehrlich pathway-derived catabolites of valine and tyrosine, respectively, were also detectable when phenylalanine, leucine, or methionine was used as the sole nitrogen source. Although 3-methylbutanol, an expected product of leucine catabolism, could not be detected in our HPLC setup, the compound was detected by ^{1}H-NMR in leucine, phenylalanine, and methionine cultures (data not shown). Conversely, none of these metabolites were detected in cultures grown with ammonium sulfate, proline, or asparagine as the nitrogen source (Table 2).

These results can be explained in two different ways. First, growth on amino acids whose catabolism involves the Ehrlich pathway may lead to coordinate induction of Ehrlich pathway enzymes with different substrate specificities. Alternatively, these amino acids may induce Ehrlich pathway enzymes with broad substrate specificities. To further investigate this phenomenon, we focused on the irreversible decarboxylase reaction.

Decarboxylation of 2-oxo acids by cell extracts of wild-type *S. cerevisiae* grown on various nitrogen sources. 2-Oxo-acid-decarboxylase activities involved in the Ehrlich pathway were analyzed in cell extracts of *S. cerevisiae* CEN.PK113-7D grown in aerobic carbon-limited chemostat cultures with different amino acids as the sole nitrogen source (Table 3). Phenylpyruvate, α-ketoisovalerate, α-ketoisocaproate, α-ketomethylvalerate, and 3-methylthio-α-ketobutyrate were selected as substrates based on the observed metabolite profiles (Table 2). Significant activities with all five substrates were detected in cultures grown with leucine, methionine, or phenylalanine as the nitrogen source (Table 3). Conversely, no activity was measured in cell extracts from cultures grown on ammonium, asparagine, or proline, in good agreement with the absence of alcohols and acids in the corresponding culture supernatants (Table 2). When activities were expressed relative to the activity with phenylpyruvate, the substrate specificity did not differ markedly as a function of the nitrogen source for growth. This suggested involvement of a single common decarboxylase activity in the catabolism of leucine, methionine, and phenylalanine (Fig. 2).

Transcript levels of TPP-dependent decarboxylase genes in wild-type *S. cerevisiae* grown on various nitrogen sources. The pyruvate-decarboxylase genes *PDC1*, *PDC5*, and *PDC6* and the related genes *THI3* and *ARO10* have all been implicated in the production of fusel alcohols and fusel acids by *S. cerevisiae* in the literature (7, 9–11, 44), but their substrate specificities and catalytic contributions remain unknown. To check whether the induction of a "broad-substrate-specificity decarboxylase activity" observed in cell extracts could be correlated with the transcriptional induction of a single gene, expression of the five decarboxylase genes was analyzed.

The levels of the *ACT1* transcript, a commonly used "loading standard" for mRNA analysis (32), were the same for all six nitrogen sources (Table 4). *PDC5*, *PDC6*, and *THI3* were transcribed at a constant, very low level. *PDC1* showed much higher transcript levels, but they did not significantly differ for the six nitrogen sources (*t* test analysis at $P < 0.01$). Only *ARO10* was differentially transcribed for the different nitrogen sources (Table 4). In cultures grown with leucine, phenylalanine, or methionine as the nitrogen source, the transcript level was at least 15-fold higher than in cultures grown with ammo-

TABLE 2. Concentrations of fusel alcohols and corresponding organic acids[a]

N source	Phenylethanol (mM)	Phenylacetate (mM)	3-Methylbutanoate (mM)	2-Methylpropanoate (mM)	3-Methylthiopropanol (mM)	3-Methylthiopropanoate (mM)	p-Hydroxyphenylacetate (mM)	p-Hydroxyphenylethanol (mM)
Ammonia	0.003 ± 0.000	0.003 ± 0.000	ND[b]	ND	ND	ND	ND	ND
Leucine	0.059 ± 0.013	0.137 ± 0.036	4.538 ± 0.351	0.310 ± 0.000	ND	ND	0.135 ± 0.031	ND
Methionine	0.225 ± 0.071	0.180 ± 0.045	ND	0.204 ± 0.000	0.757 ± 0.309	ND	0.190 ± 0.017	0.054 ± 0.025
Phenylalanine	.261 ± 0.141	9.915 ± 0.681	ND	0.161 ± 0.021	ND	ND	0.076 ± 0.020	ND
Proline	ND	ND	ND	ND	0.037 ± 0.007	ND	ND	ND
Asparagine	ND	ND	ND	ND	0.026 ± 0.004	ND	ND	ND

[a] In aerobic, glucose-limited chemostat cultures ($D = 0.10\ h^{-1}$) of *S. cerevisiae* CEN.PK 113-7D grown with different nitrogen sources. Data are presented as average ± mean deviation of metabolite quantification from two independent chemostat cultures. 3-Methylbutanol (derived from leucine), 2-methylpropanol (derived from valine), 2-methylbutanol, and 2-methylbutanoate (derived from isoleucine) were not detected by the HPLC setup used in the present study.
[b] ND, not detected.

TABLE 3. Specific activities of 2-oxo acid decarboxylation by cell extracts[a]

Nitrogen source	Specific decarboxylase activity [nmol · min^{-1} · (mg protein)$^{-1}$]				
	Phenylpyruvate (phenylalanine)	α-Ketoisovalerate (valine)	α-Ketoisocaproate (leucine)	α-Ketomethylvalerate (isoleucine)	3-Methylthio α-ketobutyrate (methionine)
Ammonia	BD[b]	BD	BD	BD	BD
Leucine	13.5 ± 0.7 (100)	4 ± 0.01 (29)	6.5 ± 0.7 (48)	4.5 ± 0.7 (33)	5.5 ± 0.6 (41)
Methionine	22.25 ± 1.8 (100)	8.5 ± 0.5 (38)	9.25 ± 0.5 (42)	5.5 ± 0.9 (25)	9 ± 0.01 (40)
Phenylalanine	67.5 ± 0.7 (100)	19 ± 0 (28)	29.5 ± 0.7 (43)	25.7 ± 3.8 (38)	22 ± 0 (32)
Proline	BD	BD	BD	BD	BD
Asparagine	BD	BD	BD	BD	BD

[a] Prepared from aerobic, glucose-limited chemostat cultures of *S. cerevisiae* CEN.PK 113-7D grown with different amino acids as the sole nitrogen source. Data are the average ± mean deviation of assays from two independent chemostat cultures. The relative 2-oxo acid activities, expressed as a percentage of phenylpyruvate activity, are in parentheses. The column headings include in parentheses the amino acid which the 2-oxo acid used as a substrate is derived from.

[b] BD, below detection limit.

nium sulfate as the nitrogen source. Moreover, cultures grown with proline or asparagine as the nitrogen source yielded the same very low *ARO10* transcript levels as ammonium sulfate-grown cultures (Table 4).

ARO10 encodes a broad-substrate-specificity 2-oxo-acid-decarboxylase in S. cerevisiae. The transcriptional regulation of *ARO10*; the similar substrate specificities of decarboxylase activities in cell extracts of leucine-, methionine-, and phenylalanine-grown cultures; and the metabolite profiles in these cultures all suggested that Aro10p is responsible for a broad-substrate-specificity decarboxylase activity involved in the production of fusel alcohols and acids. To test this hypothesis, an *S. cerevisiae* strain lacking all five TPP-dependent decarboxylase genes (CEN-PK711-7C *pdc1Δ pdc5Δ pdc6Δ thi3Δ aro10Δ ura3Δ*) was constructed. The *ura3* genotype was complemented by transformation either with the empty expression vector p426GPD (strain IMZ001) or with the same vector carrying

ARO10 under the control of the constitutive *TDH3* promoter (strain IMZ002). Strains IMZ001 and IMZ002 could not grow on glucose synthetic media as a result of the *pdc1Δ pdc5Δ pdc6Δ* genotype (15). Therefore, ethanol was used as a carbon source.

Cell extracts of the quintuple-deletion strain IMZ001, grown in aerobic, ethanol-limited chemostat cultures at a dilution rate of 0.05 h^{-1} and with ammonium sulfate as the nitrogen source, did not exhibit any decarboxylase activity (Table 5). Constitutive expression of *ARO10* in this genetic background (strain IMZ002) restored decarboxylase activity with the 2-oxo acids derived from leucine, phenylalanine, and methionine. Interestingly, no activity could be measured with pyruvate as a decarboxylase substrate (Table 5). The relative specific activities with the nonpyruvate substrates were similar to those observed in cell extracts of the reference strain CEN.PK 113-7D grown
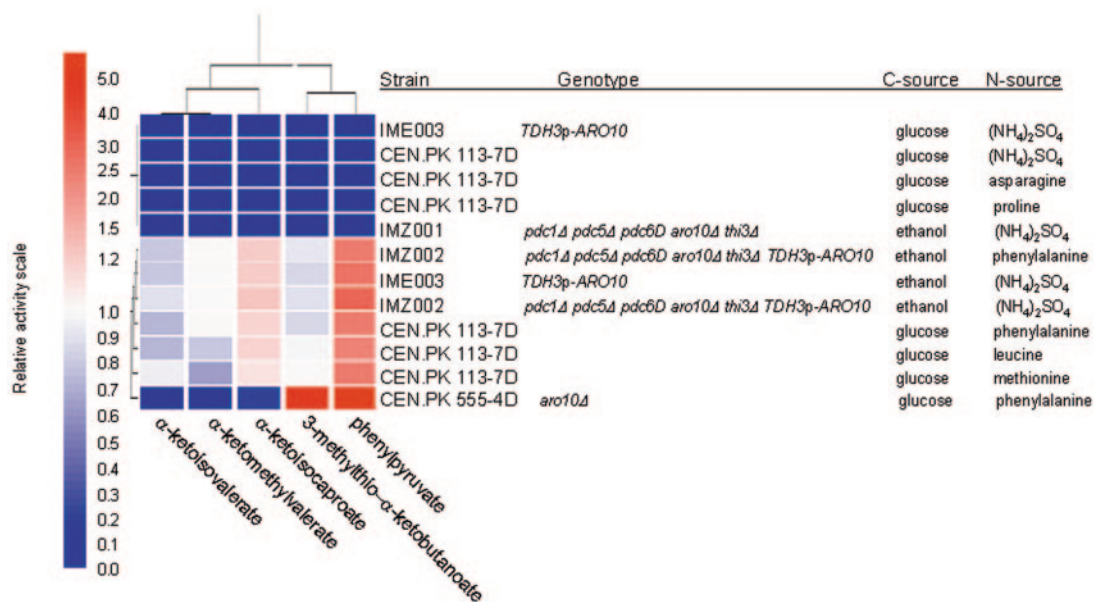

FIG. 2. Eisen representation of relative 2-oxo acid decarboxylase activity. Cell extracts of CEN.PK113-7D, CEN.PK 555-4D, IME003, IMZ001, and IMZ002 grown in aerobic carbon-limited (glucose or ethanol) chemostat cultures with different nitrogen sources were measured for 2-oxo-acid decarboxylase activity. Each cell extract was tested for conversion of phenylpyruvate, α-ketoisovalerate, α-ketoisocaproate, α-ketomethylvalerate, and 3-methylthio-α-ketobutyrate. The activity data were normalized to the mean and clustered by hierarchical clustering using Genespring (Silicon Genetics, Redwood City, CA). The so-called normalized data were displayed on a scale from 0 to 5 (see Materials and Methods).

TABLE 4. Transcript levels of genes with sequence similarity to thiamin-pyrophosphate-dependent decarboxylases in aerobic, glucose-limited chemostat cultures of *S. cerevisiae* CEN.PK113-7D grown with different amino acids as the sole nitrogen source[a]

Nitrogen source	ACT1	ARO10	PDC1	PDC5	PDC6	THI3
Ammonia	2,488 ± 81	67 ± 3	1,123 ± 147	95 ± 4	66 ± 4	92 ± 9
Leucine	2,149 ± 204	1,045 ± 167	1,311 ± 90	73 ± 7	31 ± 8	128 ± 11
Methionine	2,831 ± 624	1,335 ± 130	1,459 ± 226	87 ± 14	17 ± 3	126 ± 22
Phenylalanine	2,917 ± 575	1,996 ± 201	894 ± 319	87 ± 32	25 ± 5	109 ± 17
Proline	2,294 ± 127	37 ± 6	1,505 ± 173	81 ± 8	76 ± 8	115 ± 12
Asparagine	2,416 ± 122	61 ± 11	1,170 ± 109	64 ± 6	46 ± 8	105 ± 47

[a] Transcript levels were determined with Affymetrix Gene Chips. Data are the average ± standard deviation of three independent chemostat cultures. The *ACT1* transcript is included as a reference.

with phenylalanine, leucine, or methionine as the nitrogen source (Fig. 2 and Tables 3 and 5).

The quintuple-deletion strain IMZ001 was unable to grow in ethanol-limited chemostat cultures ($D = 0.05$ h^{-1}) when phenylalanine was the sole nitrogen source. This ability was recovered in strain IMZ002, which constitutively expresses *ARO10* from the *TDH3* promoter. Unexpectedly, decarboxylase activities in cell extracts of strain IMZ002 grown with phenylalanine as the nitrogen source were 4.5-fold higher than in cultures grown with ammonium sulfate as the nitrogen source (Table 5). Reintroduction of *ARO10* in the quintuple-deletion strain also restored the production of fusel alcohols and acids. HPLC analysis of ethanol-limited, ammonium-grown chemostat cultures of IMZ002 revealed low but significant concentrations of phenylethanol (0.11 ± 0.01 mM) and phenylacetate (0.20 ± 0.03 mM). The concentrations of these compounds were below the HPLC detection limit (0.003 mM) in chemostat cultures of the quintuple-deletion strain IMZ001. When the *ARO10*-expressing strain was grown with phenylalanine as the nitrogen source, high concentrations of phenylethanol (2.69 ± 0.06 mM) and phenylacetate (7.29 ± 0.34 mM) were observed in culture supernatants. Furthermore, low concentrations of 3-methylthiopropanol (0.67 mM) and *p*-hydroxyphenylacetate (0.16 mM) were identified, confirming the involvement of Aro10p in the synthesis of a broad range of fusel alcohols and acids in vivo.

Overexpression of *ARO10* in the isogenic reference strain CEN.PK 113-5D. To investigate whether overexpression of *ARO10* can be used to modify fusel alcohol production by wild-type *S. cerevisiae* strains, the expression vector carrying *ARO10* under the control of the *TDH3* promoter was introduced into the reference strain CEN.PK113-5D (resulting in strain IME003 [Table 1]). Surprisingly, except for pyruvate decarboxylase, no 2-oxo acid decarboxylase activity was detectable in cell extracts of this strain when it was grown in glucose-limited chemostat cultures with ammonium sulfate as the nitrogen source (Table 6). Monitoring of the *ARO10* transcript level by quantitative PCR in strain IM003 grown in a glucose-limited chemostat with ammonium sulfate as the nitrogen source revealed expression of the *TDH3*-driven construct. The level of expression was equivalent to half of the *ACT1* reference transcript signal. In the meantime, no *ARO10* transcript was detected in strain IME004 grown under similar conditions. Furthermore, decarboxylase activities in glucose-limited chemostat cultures grown with phenylalanine as the nitrogen source were the same as those of the empty-vector reference strain IME004 (Table 6). When ethanol instead of glucose was used as the carbon source, the presence of the *ARO10* expression vector did result in increased decarboxylase activities relative to an empty-vector reference strain (Table 6). These results contradict the simple view that *ARO10* encodes a fully functional decarboxylase whose expression is primarily regulated at the level of transcription.

DISCUSSION

Formation of fusel alcohols by *S. cerevisiae*. In brewery and wine fermentations, *S. cerevisiae* is responsible for the production of a variety of metabolites that contribute to flavor and aroma. Among the volatile flavor compounds, an important

TABLE 5. Substrate specificity of the *ARO10*-dependent 2-oxo-acid-decarboxylase activity in *S. cerevisiae*[a]

Substrate	Enzyme activity nmol min^{-1} (mg protein)$^{-1}$			
	IMZ0001 [(NH$_4$)$_2$SO$_4$[c]]	IMZ002 [(NH$_4$)$_2$SO$_4$[c]]	IMZ002 [phenylalanine[c]]	Ratio[b]
Phenylpyruvate	BD[d]	61.75 ± 1.71 (100)	270 ± 6.98 (100)	4.37
α-Ketoisovalerate	BD	16.75 ± 2.21 (27)	78.25 ± 4.1 (29)	4.67
α-Ketoisocaproate	BD	25 ± 0.82 (40)	118 ± 7.53 (44)	4.72
α-Ketomethylvalerate	BD	21 ± 1.63 (34)	97 ± 6.68 (36)	4.62
3-Methylthio-α-ketobutyrate	BD	18.5 ± 1.29 (30)	87.7 ± 7.69 (32)	4.74
Pyruvate	BD	BD	BD	

[a] Strain IMZ001 is *pdc1Δ pdc5Δ pdc6Δ aro10Δ thi3Δ* carrying the empty expression vector p426GPD (2μ *URA3 TDH3p*). Strain IMZ002 is the same strain carrying the plasmid pUDe001 (2μ *URA3 TDH3p-ARO10*). Both strains were grown in aerobic, ethanol-limited chemostat cultures with ammonia as the nitrogen source. Enzyme activities were assayed in cell extracts. Data are the average ± average deviation of the mean from assays of two independent chemostat cultures. The relative 2-oxo acid activities, expressed as a percentage of phenylpyruvate activity, are in parentheses.
[b] Ratio of phenylalanine versus (NH$_4$)$_2$SO$_4$.
[c] N source.
[d] BD, below detection limit.

TABLE 6. Regulation of decarboxylase activities in the reference *S. cerevisiae* strain IME004 (CEN.PK113-5D, p426GPD) and in an isogenic strain expressing a multicopy plasmid-borne *ARO10* gene from a constitutive *TDH3* promoter, strain IME003[a]

| Substrate for decarboxylase assay | IME004 [*ura3* p426GPD (*URA3*)] | | | | IME003 [*ura3* pUDe001 (*URA3 TDH3p-ARO10*)] | | | |
| | Ethanol | | Glucose | | Ethanol | | Glucose | |
	$(NH_4)_2SO_4$	Phenylalanine	$(NH_4)_2SO_4$	Phenylalanine	$(NH_4)_2SO_4$	Phenylalanine	$(NH_4)_2SO_4$	Phenylalanine
Phenylpyruvate	BD[b]	64 ± 1.2	BD	67.5 ± 0.7	35 ± 5.7	76.5 ± 1.5	BD	82.75 ± 26.73
α-Ketoisovalerate	BD	17 ± 2.2	BD	19 ± 0	19 ± 0	21.75 ± 1.7	BD	22.4 ± 6.4
α-Ketoisocaproate	BD	28 ± 3.1	BD	29.5 ± 0.7	15 ± 1.4	30.5 ± 3	BD	31.5 ± 9.8
α-Ketomethylvalerate	BD	23 ± 0.7	BD	25.7 ± 3.8	12.5 ± 0.7	28 ± 4.0	BD	28.3 ± 13.6
3-Methylthio-α-ketobutyrate	BD	20.5 ± 1.2	BD	22 ± 0	11 ± 0	23.5 ± 1.2	BD	24.25 ± 9.1

[a] Both strains were grown in aerobic, carbon-limited chemostat cultures with glucose or ethanol as a carbon source and ammonia or phenylalanine as a carbon source and ammonia or phenylalanine as a nitrogen source. Enzyme activities were assayed in cell extracts of independent duplicate cultures and are expressed as $nmol \cdot min^{-1} \cdot (mg\ protein)^{-1}$. Data are presented as average ± average deviation of the mean of two chemostat cultures.
[b] BD, below detection limit.

class consists of higher alcohols that are less volatile than ethanol (18). These higher alcohols are derived from the carbon skeletons of amino acids, which can in theory be synthesized de novo but in brewery and wine fermentations are generally taken up from the wort or grape must. It is commonly accepted that branched-chain (9–11) and aromatic (7, 44) amino acid-derived alcohols originate from the Ehrlich pathway (12) (Fig. 1). Our results support the notion that this pathway is also involved in the production of 3-methylthiopropanol (methionol) and 3-methylthiopropanoate from methionine. These sulfur-containing compounds are relevant to the production of alcoholic beverages. Methionol, which has a raw-potato odor, is commonly measured in wine and is known to negatively affect white wine and red wine aroma above 0.6 mg/liter and 2 to 3 mg/liter, respectively (3) (Table 1).

Our results indicated that induction of an Ehrlich pathway for catabolism of one amino acid leads to the formation of significant amounts of fusel alcohols and acids from other amino acids. This suggested that conversion of branched-chain, aromatic, and sulfur-containing amino acids to the corresponding fusel alcohols and acids, via an Ehrlich pathway, involves common broad-substrate-specificity enzyme activities. Furthermore, as our experiments were performed with synthetic media to which only single amino acids were added, these results indicated that the decarboxylase activity involved in the Ehrlich pathway could compete for 2-oxo acids with the transaminases involved in de novo amino acid biosynthesis. The chemostat conditions used in this study were designed to reveal the molecular nature of the decarboxylase step of the Ehrlich pathway. Although these conditions are different from typical alcoholic fermentation processes, the conclusion drawn about the role of *ARO10* is relevant for interpreting the patterns of flavor production in wine and beer fermentation.

Aro10p is involved in a broad-substrate-specificity Ehrlich pathway decarboxylase activity. Transcript analysis demonstrated that the induction of Ehrlich pathway activity by the amino acids leucine, phenylalanine, and methionine coincided with the transcriptional up-regulation of *ARO10*, but not with that of the other four genes encoding (putative) thiamine pyrophosphate-dependent decarboxylases. Indeed, overexpression of *ARO10* in a strain in which the five chromosomal decarboxylase genes had been deleted was sufficient to restore a broad-substrate-specificity decarboxylase activity. The substrate specificity profile of this strictly *ARO10*-dependent ac-

tivity was the same as those of the activities induced by leucine, phenylalanine, and methionine in wild-type cells.

Previous research in *S. cerevisiae* with *aro10* null mutants has indicated the presence of an *ARO10*-independent decarboxylase activity (44). This alternative activity has been reported to require the simultaneous expression of at least one of the three pyruvate decarboxylase genes (*PDC 1*, *-5*, and *-6*) and the putative decarboxylase gene *THI3*. Based on previous work by Dickinson and coworkers, the last gene has also been implicated in the decarboxylation of the 2-oxo acids derived mainly from leucine (11) and to a lesser extent isoleucine (10). This *ARO10*-independent 2-oxo acid decarboxylase activity exhibited a completely different substrate specificity profile. In particular, the decarboxylase activity observed in cultures of an *aro10* null mutant grown with phenylalanine as the nitrogen source showed no activity with α-ketoisovalerate and α-ketomethylvalerate as the substrate (Fig. 2, strain CEN.PK 555-4D).

The results described here support the notion that the *ARO10*-dependent, broad-substrate-specificity decarboxylase is primarily responsible for the Ehrlich pathway decarboxylation reaction in wild-type *S. cerevisiae*. The molecular basis and substrate specificity of the *ARO10*-independent activity that is detected in *aro10* null mutants (44) (Table 5), as well as its possible involvement in (off-)flavor production by wild-type strains, require further research.

Transcriptional regulation of *ARO10*. Previous work has shown that transcription of *ARO10* is induced by tryptophan (24) and phenylalanine (44) and is dependent on the transcriptional regulator Aro80p (24). Other studies (7, 9–11) suggested that expression of *ARO10* might also be up-regulated by valine and isoleucine, based on metabolite profiling; however, this conclusion was not backed up by expression analysis. Our results clearly show that *ARO10* expression was strongly up-regulated in the presence of leucine and methionine (Table 3), consistent with its proposed role as a broad-substrate-specificity decarboxylase.

Further analysis of the transcriptome data revealed that *ARO9* (aromatic amino transferase II) (23) was coexpressed with *ARO10* in cultures grown with different nitrogen sources (data not shown). This suggested that the transaminase activity of Aro9p might not be restricted to aromatic amino acids (24) but, similar to the Aro10p-dependent decarboxylase activity, might have a broad substrate specificity. It remains to be in-

vestigated whether and to what extent Aro80p is involved in the transcriptional up-regulation of *ARO9* and *ARO10* by the nonaromatic amino acids leucine and methionine. This question could not be resolved by the microarray analyses, since *ARO80* transcript levels were extremely low and did not differ significantly for the nitrogen sources studied (data not shown). Further research with *aro80* null strains is required to investigate whether the regulatory role of Aro80p extends beyond aromatic amino acid metabolism expression control or, alternatively, another regulatory protein or proteins control the upregulation of *ARO9* and *ARO10* in leucine- and methionine-grown cultures. A comprehensive discussion of the genome-wide transcriptional responses of *S. cerevisiae* to the six nitrogen sources used in this study will be published elsewhere (V. M. Boer, S. L. Tai, Z. Vuralhan, Y. Afrifin, M. C. Walsh, M. D. W. Piper, J. H. de Winde, J.-M. Daran, and J. T. Pronk, unpublished data).

Involvement of other factors in the activity and regulation of Aro10p. Earlier works (7, 9–11, 15, 40, 44) on the decarboxylation of branched-chain and aromatic 2-oxo acid decarboxylation were based on the implicit assumption that single proteins (e.g., Aro10p and/or Thi3p) would act as thiamine pyrophosphate-dependent decarboxylase enzymes. While the present study proves that Aro10p plays a key role in broad-substrate-specificity decarboxylase activity, it also provides several clear indications that additional factors are involved in this activity and its regulation.

Our attempt to overexpress *ARO10* under the control of the *TDH3* promoter in order to uncouple its expression from environmental parameters, such as the presence of phenylalanine, yielded unexpected results. In cultures grown with ammonium sulfate as the nitrogen source, the *TDH3p-ARO10* construct yielded activity in ethanol-grown cultures but, surprisingly, not when glucose was the carbon source. This unexpected dependency on the carbon source was independent of the expression of the other four decarboxylase genes. As the *TDH3* promoter is known to give very high transcript levels in glucose- as well as ethanol-grown cultures, this observation suggests that transcription of the *ARO10* gene is not sufficient to yield an active broad-substrate-specificity decarboxylase activity. Furthermore, in ethanol-grown cultures of the "*ARO10* constitutive" strains, addition of phenylalanine to culture media caused a strong increase in the broad-substrate-specificity decarboxylase activities in cell extracts.

These observations may indicate that the functional expression of the *ARO10* gene is regulated at a posttranscriptional level in a carbon and nitrogen source-dependent manner. Alternatively, the catalytic activity and/or stability of Aro10p may require the presence of one or more additional proteins whose expression is carbon and nitrogen source dependent.

Recent protein interactome studies based on the two-hybrid approach (25) identified two potential Aro10p interaction partners. Fit2p is possibly involved in iron uptake (33, 36), and Ena5p is an ATP-driven sodium transporter, a member of the Na^+-transporting ATPase family in the superfamily of P-type ATPases (5, 26). Taking into account the subcellular localization of Fit2p and Ena5p (the cell wall and plasma membrane, respectively), it is difficult to envision them as key factors in controlling the activity or stability of Aro10p. Of these two putative partners, only *FIT2* would show an expression profile that would corroborate our assumption (data not shown).

The present study has clearly established the importance of Aro10p in the key decarboxylation step of the Ehrlich pathway. At the same time, it has raised new and important questions about the additional factors involved in the molecular composition, posttranscriptional regulation, and/or stability of the Aro10p-dependent decarboxylase activity. These questions need to be resolved before strategies can be devised to rationally modify the production of volatile flavor compounds by *S. cerevisiae* in beverages and fine-chemical production, e.g., via genetic modification of in vivo decarboxylase activity. Purification and characterization of the broad-substrate-specificity decarboxylase from cell extracts is likely to be essential to resolve the outstanding issues.

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