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Substrate Specificity of Thiamine Pyrophosphate-Dependent 2-Oxo-Acid Decarboxylases in *Saccharomyces cerevisiae*

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Fusel alcohols are precursors and contributors to flavor and aroma compounds in fermented beverages, and some are under investigation as biofuels. The decarboxylation of 2-oxo acids is a key step in the Ehrlich pathway for fusel alcohol production. In *Saccharomyces cerevisiae*, five genes share sequence similarity with genes encoding thiamine pyrophosphate-dependent 2-oxo-acid decarboxylases (2ODCs). *PDC1*, *PDC5*, and *PDC6* encode differentially regulated pyruvate decarboxylase isoenzymes; *ARO10* encodes a 2-oxo-acid decarboxylase with broad substrate specificity, and *THI3* has not yet been shown to encode an active decarboxylase. Despite the importance of fusel alcohol production in *S. cerevisiae*, the substrate specificities of these five 2ODCs have not been systematically compared. When the five 2ODCs were individually overexpressed in a *pdc1*Δ *pdc5*Δ *pdc6*Δ *aro10*Δ *thi3*Δ strain, only Pdc1, Pdc5, and Pdc6 catalyzed the decarboxylation of the linear-chain 2-oxo acids pyruvate, 2-oxo-butanoate, and 2-oxo-pentanoate in cell extracts. The presence of a Pdc isoenzyme was also required for the production of *n*-propanol and *n*-butanol in cultures grown on threonine and norvaline, respectively, as nitrogen sources. These results demonstrate the importance of pyruvate decarboxylases in the natural production of *n*-propanol and *n*-butanol by *S. cerevisiae*. No decarboxylation activity was found for Thi3 with any of the substrates tested. Only Aro10 and Pdc5 catalyzed the decarboxylation of the aromatic substrate phenylpyruvate, with Aro10 showing superior kinetic properties. Aro10, Pdc1, Pdc5, and Pdc6 exhibited activity with all branched-chain and sulfur-containing 2-oxo acids tested but with markedly different decarboxylation kinetics. The high affinity of Aro10 identified it as a key contributor to the production of branched-chain and sulfur-containing fusel alcohols.

Pyruvate decarboxylase (PDC) catalyzes the thiamine pyrophosphate (TPP)-dependent decarboxylation of pyruvate to acetaldehyde. In *Saccharomyces cerevisiae*, PDC is not only a key enzyme of alcoholic fermentation but is also required for the synthesis of cytosolic acetyl coenzyme A (acetyl-CoA) (15, 16), a key precursor for the synthesis of lipids and lysine. Three PDC isoenzymes are encoded by the *S. cerevisiae PDC1*, *PDC5*, and *PDC6* genes (25). Pdc1, the main isoenzyme, is highly expressed under most conditions, while *PDC5* exhibits tight transcriptional control, with high expression levels in the absence of a functional *PDC1* gene (48), under nitrogen-limited conditions (3–5, 53), and under conditions of thiamine limitation (40). *PDC1* and *PDC5* expressions are subject to autoregulation (24) and require the transcription factor Pdc2 (23). The third PDC isoenzyme, Pdc6, has a very low content of sulfur-containing amino acids and is highly expressed in sulfur-limited cultures (4, 53). This transcriptional regulation of *PDC6* has been interpreted as a "sulfur economy" response (4, 14, 53).

The *ARO10* and *THI3* genes show sequence homology to the *PDC* genes but do not encode pyruvate decarboxylases. However, the conversion of pyruvate to acetaldehyde is not the only physiologically relevant TPP-dependent decarboxylation of a 2-oxo acid in *S. cerevisiae*. In the Ehrlich pathway, several 2-oxo acids that derive from the transamination of amino acids are decarboxylated, and the resulting aldehydes are reduced to the corresponding "fusel" alcohols (60, 61). Alternatively, under aerobic conditions, the aldehydes can be oxidized to the corresponding "fusel acids" (5). Several hypotheses have been postulated for the physiological relevance of fusel alcohol formation in *S. cerevisiae*, including a role as a redox sink, in the detoxification of 2-oxo acids, and in the generation of a thermodynamic "pull" to facilitate the transfer of amino groups from amino acids during nitrogen-limited growth (22). In addition, several fusel alcohols and acids have been shown to act as signaling molecules in yeasts. For example, phenylethanol and indole-3-acetic acid trigger morphological changes in *S. cerevisiae* (8, 46).

Fusel alcohol production is of considerable commercial importance. Fusel alcohols and their esters are important flavor constituents of fermented foods and beverages (52). Furthermore, phenylethanol, which has a characteristic roselike flavor, is intensively used in cosmetics and fragrances (13). Finally, the production of several fusel alcohols, including isobutanol, is under intensive study to explore their possible application as transport fuels, because their physical and chemical properties offer advantages over those of ethanol (1, 2).

Characterizations of deletion mutants indicated that Aro10 can catalyze the decarboxylation of several aromatic and branched-chain 2-oxo acids (60, 61), but no evidence has so far been found for a catalytic activity of Thi3. Therefore, the observation that *aro10*Δ mutants are still able to decarboxylate phenylpyruvate indicates that at least one of the PDCs has a substrate spectrum that allows the decarboxylation of substrates other than

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pyruvate. The kinetic properties of PDCs originating from different microorganisms were compared previously (41). However, a systematic comparison of the substrate specificity of the individual 2-oxo-acid decarboxylases in *S. cerevisiae* is not available, and previous biochemical studies of PDC in this yeast, which indicated a broad substrate specificity (33, 51), were likely to have been based on mixtures of the three Pdc isoenzymes (34), possibly contaminated with Aro10 (12).

Knowledge of the substrate specificities of individual 2-oxo-acid decarboxylases in *S. cerevisiae* is essential for an understanding of the regulation of flavor and aroma production and for the metabolic engineering of this yeast for the production of individual fusel alcohols. The goal of the present study is to assess the substrate specificities of Pdc1, Pdc5, Pdc6, Aro10, and Thi3 for different 2-oxo acids. To this end, the five structural genes encoding these proteins were individually expressed in a *pdc1*Δ *pdc5*Δ *pdc6*Δ *aro10*Δ *thi3*Δ strain, followed by an analysis of substrate specificity and decarboxylation kinetics in cell extracts. The role of the *PDC* genes in the production of *n*-propanol and *n*-butanol by *S. cerevisiae* was further investigated by an *in vivo* analysis of product formation in batch cultures grown on different nitrogen sources.

MATERIALS AND METHODS

Construction of plasmids and strains. The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Genomic DNA of reference strains CEN.PK113-7D and S288C was prepared as described previously (7).

2-Oxo-acid decarboxylase overexpression constructs. The *PDC1* gene was amplified from genomic DNA of S288C by using primers *PDC1* Fw and *PDC1* Rv (Table 2). The resulting PCR product was cloned into pENTR/D-TOPO, resulting in pENTR/D-TOPO-*PDC1*.3, and then cloned into destination plasmid pVV214 (56) via the LR reaction by using Gateway technology (Invitrogen, Carlsbad, CA), resulting in pEXP214-*PDC1*.3 (Table 3). The *PDC6* gene was amplified from genomic DNA of strain S288C by using primers *PDC6* Fw and *PDC6* Rv. The resulting PCR product was cloned into pENTR/D-TOPO, resulting in pENTR/D-TOPO-*PDC6*.2, and then cloned into pVV214 via the LR reaction by using Gateway technology (Invitrogen), resulting in pEXP214-*PDC6*.2 (Table 3). The *THI3* gene was amplified from genomic DNA of strain S288C by using primers *THI3* Fw HindIII and *THI3* Rv XhoI and then cloned into pCR-Blunt-TOPO, resulting in TOPO-HindIII-*THI3*-XhoI-1. Both the TOPO-HindIII-*THI3*-XhoI-1 and p426-GPD vectors (42) were digested with HindIII and XhoI, purified from gel, and ligated into the resulting plasmid, pUDe005 (Table 3). The *PDC5* gene was amplified from genomic DNA of strain S288C by using primers *PDC5* Fw SpeI and *PDC5* Rv XhoI. Both the purified PCR product and the p426-GPD vector were digested with SpeI and XhoI, purified from gel, and ligated, resulting in plasmid pUDe002 (Table 3).

Construction of strain CEN.PK182 (*pdc1*Δ *pdc5*Δ *pdc6*Δ). The strategy used was to first generate single deletion mutants of each pyruvate decarboxylase gene (*PDC1*, *PDC5*, and *PDC6*). The gene deletions were performed by the integration of a *loxP-kan-loxP* cassette by homologous recombination at the locus of the corresponding *PDC* gene using a short flanking homology PCR method described previously (62). The deletion cassettes were amplified by using pUG6 (20) as a template and specific primers (Table 2). All deletions were constructed in diploid strain CEN.PK122. G418-resistant transformants were analyzed by tetrad dissection (7), and G418-resistant segregants were further analyzed by diagnostic PCR (see Table S1 in the supplemental material).

Thereafter, haploid strains were crossed as follows: *pdc1*::*loxP-kan-loxP* × *pdc6*::*loxP-kan-loxP* and *pdc5*::*loxP-kan-loxP* × *pdc6*::*loxP-kan-loxP*. Tetrads showing a nonparental distribution segregation for the G418 phenotype were further verified by diagnostic PCR, resulting in the haploid double deletions *pdc1*::*loxP-kan-loxP pdc6*::*loxP-kan-loxP* and *pdc5*::*loxP-kan-loxP pdc6*::*loxP-kan-loxP*. Both double deletions were subsequently crossed, and tetrads were dissected in YEP–3% ethanol–0.05% glucose medium (10 g · liter^{-1} yeast extract, 20 g · liter^{-1} peptone, 3% ethanol, 0.05% glucose) (7). Thereafter, segregants showing a nonparental distribution segregation for the nongrowing phenotype in 2% glucose medium were further verified by diagnostic PCR, resulting in the triple deletion strain (*pdc1*::*loxP-kan-loxP pdc5*::*loxP-kan-loxP pdc6*::*loxP-kan-loxP*). To eliminate the *kan* marker(s), the triple *pdc* deletion strain was

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

Strain	Genotype	Source or reference
CEN.PK113-7D	*MAT***a** MAL2-8c *SUC2*	P. Kötter[a]
CEN.PK122	*MAT***a**/*MAT*α	P. Kötter[a]
CEN.PK555-4A	*MAT***a** *URA3 aro10*::*loxP-kan-loxP*	61
CEN.PK555-4D	*MAT*α *URA3 aro10*::*loxP-kan-loxP*	This study
CEN.PK182	*MAT***a** *URA3 pdc1*Δ *pdc5*Δ *pdc6*Δ	This study
CEN.PK113-13D	*MAT*α *ura3-52*	P. Kötter[a]
CEN.PK553-1A	*MAT*α *URA3 thi3*::*loxP-kan-loxP*	This study
CEN.PK707	*MAT*α/*MAT***a** *URA3/ura3-52 pdc1*Δ/*PDC1 pdc5*Δ/*PDC5 pdc6*Δ/*PDC6*	This study
CEN.PK608	*MAT*α/*MAT***a** *URA3/ura3-52 pdc1*Δ/*PDC1 pdc5*Δ/*PDC5 pdc6*Δ/*PDC6 THI3/thi3*::*loxP-kan-loxP*	This study
CEN.PK707-4A	*MAT***a** *ura3-52 pdc1*Δ *pdc5*Δ *pdc6*Δ	This study
CEN.PK608-4B	*MAT*α *URA3 pdc1*Δ *pdc5*Δ *pdc6*Δ *thi3*::*loxP-kan*::*loxP*	This study
CEN.PK709	*MAT*α/*MAT***a** *URA3/ura3-52 pdc1*Δ/*pdc1*Δ/*pdc5*Δ/*pdc5*Δ *pdc6*Δ/*pdc6*Δ *THI3/thi3*::*loxP-kan-loxP*	This study
CEN.PK709-4D	*MAT***a** *ura3-52 pdc1*Δ *pdc5*Δ *pdc6*Δ *thi3*::*loxP-kan-loxP*	This study
CEN.PK609	*MAT*α/*MAT***a** *URA3/ura3-52 pdc1*Δ/*PDC1 pdc5*Δ/*PDC5 pdc6*Δ/*PDC6 ARO10/aro10*::*loxP-kan-loxP*	This study
CEN.PK609-14B	*MAT*α *URA3 pdc1*Δ *pdc5*Δ *pdc6*Δ *aro10*::*loxP-kan-loxP*	This study
CEN.PK710	*MAT*α/*MAT***a** *URA3/ura3-52 pdc1*Δ/*pdc1*Δ/*pdc5*Δ/*pdc5*Δ *pdc6*Δ/*pdc6*Δ *ARO10/aro10*::*loxP-kan-loxP*	This study
CEN.PK710-2B	*MAT*α *ura3-52 pdc1*Δ *pdc5*Δ *pdc6*Δ *aro10*::*loxP-kan-loxP*	This study
CEN.PK711	*MAT*α/*MAT***a** *ura3-52/ura3-52 pdc1*Δ/*pdc1*Δ/*pdc5*Δ/*pdc5*Δ *pdc6*Δ/*pdc6*Δ *THI3/thi3*::*loxP-kan-loxP ARO10/aro10*::*loxP-kan-loxP*	This study
CEN.PK711-7C	*MAT***a** *ura3-52 pdc1*Δ *pdc5*Δ *pdc6*Δ *aro10*::*loxP-kan-loxP thi3*::*loxP-kan-loxP*	60
IMZ001	*MAT***a** *ura3-52 pdc1*Δ *pdc5*Δ *pdc6*Δ *aro10*::*loxP-kan-loxP thi3*::*loxP-kan-loxP* p426GPD (*URA3*)	60
IMZ002	*MAT***a** *ura3-52 pdc1*Δ *pdc5*Δ *pdc6*Δ *aro10*::*loxP-kan-loxP thi3*::*loxP-kan-loxP* pUDe001 (*URA3* $TDH3_{pr}$-*ARO10*-$CYC1_{ter}$)	60
IMZ024	*MAT***a** *ura3-52 pdc1*Δ *pdc5*Δ *pdc6*Δ *aro10*::*loxP-kan-loxP thi3*::*loxP-kan-loxP* pUDe002 (*URA3* $TDH3_{pr}$-*PDC5*-$CYC1_{ter}$)	This study
IMZ025	*MAT***a** *ura3-52 pdc1*Δ *pdc5*Δ *pdc6*Δ *aro10*::*loxP-kan-loxP thi3*::*loxP-kan-loxP* pUDe005 (*URA3* $PGK1_{pr}$-*THI3*-$CYC1_{ter}$)	This study
IMZ030	*MAT***a** *ura3-52 pdc1*Δ *pdc5*Δ *pdc6*Δ *aro10*::*loxP-kan-loxP thi3*::*loxP-kan-loxP* pEXP214-*PDC1*.3 (*URA3* $PGK1_{pr}$-*PDC1*-$CYC1_{ter}$)	This study
IMZ031	*MAT***a** *ura3-52 pdc1*Δ *pdc5*Δ *pdc6*Δ *aro10*::*loxP-kan-loxP thi3*::*loxP-kan-loxP* pEXP214-*PDC6*.2 (*URA3* $PGK1_{pr}$-*PDC6*-$CYC1_{ter}$)	This study
IMI078	*MAT***a** *URA3 pdc1*Δ *pdc5*Δ *pdc6*Δ $mth1^{\Delta T}$	Bart Oud[b]

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TABLE 2 Oligonucleotide primers used in this study

Primer and use	Sequence (5′→3′)
Overexpression	
THI3 Fw HindIII	GGTAAGCTTATGAATTCTAGCTATACACAGAGATATGC
THI3 Rv XhoI	GGCCTCGAGTCAGTATCCAACTTGATTTTTTTTTAGAAGTGGTTGG
PDC5 Fw SpeI	GCCACTAGTATGTCTGAAATAACCTTAGGTAAATATTTATT
PDC5 Rv XhoI	GGCCTCGAG TTATTGTTTAGCGTTAGTAGCGGCAGTCAATT
PDC6 Fw	CACCGCCAACAAAATGTCTGAAATTACTCTTG
PDC6 Rv	TTATTGTTTGGCATTTGTAGCGGCAGTCAATTGC
PDC1 Fw	CACCATGTCTGAAATTACTTTGGGTAAATATTTGTTC
PDC1 Rv	TTATTGCTTAGCGTTGGTAGCAGCAGTC
Gene deletion	
PDC1-S1	TTCTACTCATAACCTCACGCAAAATAACACAGTCAAATCACAGCTGAAGCTTCGTACGC
PDC1-S2	AATGCTTATAAAACTTTAACTAATAATTAGAGATTAAATCGCATAGGCCACTAGTGGATCTG
PDC5-S1	ATCAATCTCAAAGAGAACAACACAATACAATAACAAGAAGCAGCTGAAGCTTCGTACGC
PDC5-S2	AAAATACACAAACGTTGAATCATGAGTTTTATGTTAATTAGCATAGGCCACTAGTGGATCTG
PDC6-S1	TAAATAAAAAACCCACGTAATATAGCAAAAACATATTGCCCAGCTGAAGCTTCGTACGC
PDC6-S2	TTTATTTGCAACAATAATTCGTTTGAGTACACTACTAATGGCATAGGCCACTAGTGGATCTG
THI3-S1	TCTAGCTATACACAGAGATATGCACTGCCGAAGTGTATAGCAGCTGAAGCTTCGTACGC
THI3-S2	ATTTACTGCGCTAGAATTTTCGTTCTCCTCTTGCACTTGTGCATAGGCCACTAGTGGATCTG
ARO10-S1	ATGGCACCTGTTACAATTGAAAAGTTCGTAAATCAAGAAGCAGCTGAAGCTTCGTACGC
ARO10-S2	AATATTGCACTTCAGAATGGTGCTCAGTTCTTGGATTGTCGCATAGGCCACTAGTGGATCTG

transformed with plasmid pSH65 expressing the *cre* recombinase gene from phage P1 (19, 20). After plasmid loss, the resulting strain was named CEN.PK182 (*pdc1*Δ *pdc5*Δ *pdc6*Δ).

Construction of quintuple mutant strain CEN.PK711-7C (*pdc1*Δ *pdc5*Δ *pdc6*Δ *aro10*Δ *thi3*Δ). The preliminary single *ARO10* and *THI3* deletion strains CEN.PK553-1A (*MAT*α *thi3*Δ) and CEN.PK555-4D (*MAT*α *aro10*Δ) were generated as described above for the *PDC* genes (Table 3). Quintuple deletion strain CEN.PK711-7C was then constructed in three phases (Fig. 1).

In order to introduce an impaired *URA3* allele into triple pyruvate decarboxylase mutant strain CEN.PK182 (*pdc1*Δ *pdc5*Δ *pdc6*Δ), this strain was crossed with strain CEN.PK113-13D (*MAT*α *ura3-52*). The resulting diploid strain, CEN.PK707, was subsequently sporulated and dissected, and *MAT***a** strain CEN.PK707-4A, which combined the *pdc1*Δ *pdc5*Δ *pdc6*Δ and *ura3-52* mutations, was selected.

Two additional strains were constructed. On the one hand, strain CEN.PK608-4B was obtained after the crossing of CEN.PK182 and CEN.PK553-1A (*MAT*α *thi3*Δ) and the dissection of the tetrads of the resulting diploid strain, CEN.PK608. On the other hand, strain CEN.PK609-14B was obtained after the crossing of CEN.PK182 and CEN.PK555-4D (*MAT*α *aro10*Δ) and the dissection of the tetrads of the resulting diploid strain, CEN.PK609.

Furthermore, to combine the triple *pdc*Δ mutation with the *thi3* deletion, strain CEN.PK707-4A (*MAT***a** *ura3-52 pdc1*Δ *pdc5*Δ *pdc6*Δ) was crossed with strain CEN.PK608-4B (*MAT*α *pdc1*Δ *pdc5*Δ *pdc6*Δ *thi3*Δ); the resulting diploid strain, CEN.PK709, was then sporulated and dissected; and haploid *MAT***a** strain CEN.PK709-4D was selected for the final cross. To combine the triple *pdc* mutation with the *aro10* deletion, strain CEN.PK707-4A (*MAT***a** *ura3-52 pdc1*Δ *pdc5*Δ *pdc6*Δ) was crossed with strain CEN.PK609-14B (*MAT*α *pdc1*Δ *pdc5*Δ *pdc6*Δ *aro10*Δ); the resulting diploid strain, CEN.PK710, was then sporulated and dissected; and haploid *MAT*α strain CEN.PK710-2B was selected for the final cross.

To obtain the final quintuple *pdc1*Δ *pdc5*Δ *pdc6*Δ *thi3*Δ *aro10*Δ strain CEN.PK711-7C, strains CEN.PK709-4D and CEN.PK710-2B were first crossed to form diploid strain CEN.PK711. Subsequently, the diploid strain was sporulated and dissected, and the haploid with the correct phenotype, CEN.PK711-7C, was then selected.

The correct genotypes of all the strains carrying single or multiple disrupted 2-oxo-acid decarboxylases were confirmed by PCR. The complete PCR scheme used for confirmation can be found in Table S1 in the supplemental material.

Strain CEN.PK711-7C was then transformed with different 2-oxo-decarboxylase expression vectors (Table 3), and transformation was carried out according to the lithium acetate (LiAc)/single-stranded DNA (ssDNA) method (18).

Chemostat cultivation. *S. cerevisiae* strains were grown in aerobic ethanol-limited chemostat cultures on a synthetic medium containing (per liter of demineralized water) 5 g $(NH_4)_2SO_4$ or 10 g phenylalanine, 3 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 5.7 g of ethanol, 1 ml of trace element solution, 1 ml of vitamin solution, and 8% of antifoam-C emulsion (Sigma-Aldrich, Zwijndrecht, Netherlands). The absence of $(NH_4)_2SO_4$

TABLE 3 Plasmids used in this study

Plasmid	Characteristic(s)	Source or reference
pCR-Blunt-TOPO		Invitrogen
TOPO-HindIII-*THI3*-XhoI-1		This study
pENTR/D-TOPO	Gateway entry clone	Invitrogen
pENTR/D-TOPO-*PDC1*.3	Gateway entry clone; *PDC1*	This study
pENTR/D-TOPO-*PDC6*.2	Gateway entry clone; *PDC6*	This study
pSH65	*URA3 ARSH4 CEN6* Tn*5-ble* $GAL1_{pr}$::*cre*::$CYC1_{ter}$	19
pVV214	Gateway destination vector (*URA3* 2μm $PGK1_{pr}$-$CYC1_{ter}$)	56
p426-GPD	*URA3* 2μm $TDH3_{pr}$-$CYC1_{ter}$	42
pUDe001	*URA3* 2μm $TDH3_{pr}$-*ARO10*-$CYC1_{ter}$	60
pUDe002	*URA3* 2μm $TDH3_{pr}$-*PDC5*-$CYC1_{ter}$	This study
pUDe005	*URA3* 2μm $TDH3_{pr}$-*THI3*-$CYC1_{ter}$	This study
pEXP214-PDC1.3	*URA3* 2μm $PGK1_{pr}$-*PDC1*-$CYC1_{ter}$	This study
pEXP214-PDC6.2	*URA3* 2μm $PGK1_{pr}$-*PDC6*-$CYC1_{ter}$	This study

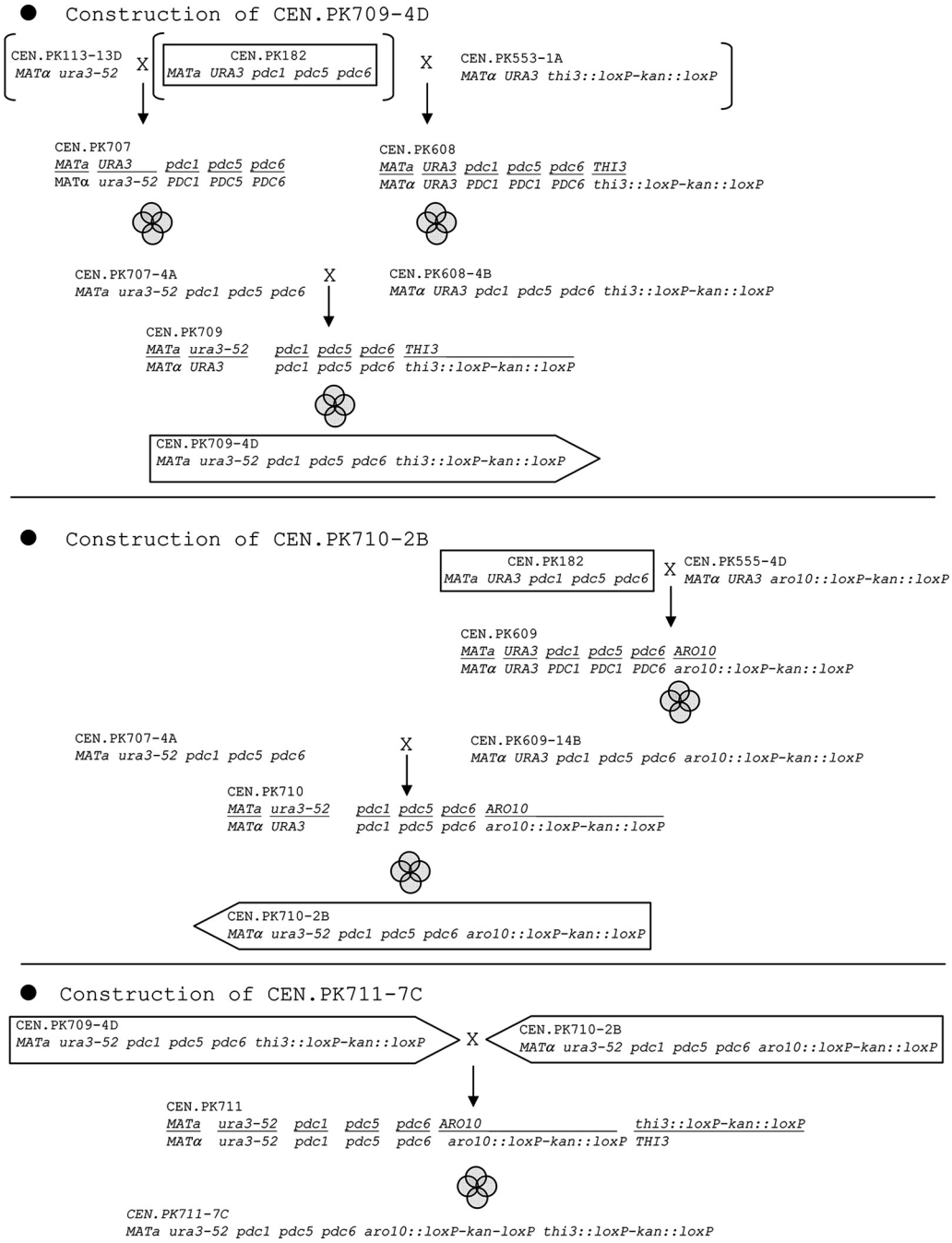

FIG 1 Scheme of the construction of quintuple 2-oxo-acid decarboxylase deletion strain CEN.PK711-7C. X represents a cross between a *MAT***a** haploid strain and a *MAT*α haploid strain, and ⊗ represents the sporulation and dissection of a diploid strain.

in phenylalanine-containing medium was compensated for by replacing it with an equimolar concentration of K_2SO_4. Trace element and vitamin solutions were prepared as described previously (59). Chemostat cultivation was performed in 2-liter bioreactors (Applikon, Schiedam, Netherlands), with a working volume of 1 liter and a dilution rate of 0.05 h^{-1}, as described previously (60). Chemostat cultures were assumed to be in the steady state after at least 5 volume changes and when the culture dry weight and off-gas CO_2 analyses differed by less than 2% over two consecutive volume changes.

Shake flask cultivation. Growth rate experiments were performed at 30°C with 100-ml shake flasks containing 20 ml of synthetic medium with 20 g · liter^{-1} glucose and 3 g · liter^{-1}ethanol. When 5 g · liter^{-1} of phenylalanine, threonine, or norvaline was used as the sole nitrogen source, the medium was filter sterilized, and the absence of $(NH_4)_2SO_4$ was compensated for by the addition of an equimolar amount of K_2SO_4 (58). Analysis of alcohols was done with a gas chromatograph (Interscience Focus, Breda, Netherlands) equipped with an HP-Innowax column (Agilent, Middelburg, Netherlands).

Preparation of cell extracts. For the preparation of cell extracts of *S. cerevisiae*, culture samples were harvested by centrifugation, washed twice with 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA, 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 and 8-μm peak-to-peak amplitude; Wolf Laboratories Ltd., Pocklington, United Kingdom). Unbroken cells and debris were removed by centrifugation at 4°C (20 min at 36,000 × *g*). The purified cell extract was then used for enzyme assays.

2-Oxo-acid decarboxylase assays. Pyruvate, 2-oxo-butanoate, and 2-oxo-pentanoate decarboxylase activities were assayed at 30°C, immediately after the preparation of cell extracts, by using a Tecan GENios Pro (Tecan, Giessen, Netherlands). The assay mixture contained, in a total volume of 300 μl, 40 mM imidazole-HCl buffer (pH 6.5), 0.2 mM thiamine pyrophosphate, 0.15 mM NADH, 88 U · ml^{-1} alcohol dehydrogenase (Sigma-Aldrich), 5 mM $MgCl_2$, and cell extract. The reaction, which was monitored as the decrease of the absorbance at 340 nm, was started by the addition of the substrate to the mixture. Reaction rates were linearly proportional to the amount of cell extract added. Measurements for calculations of K_m and V_{max} values were performed by using substrate concentrations ranging from 0 to 50 mM for pyruvate, 0 to 5 mM for 2-oxo-butanoate, and 0 to 8 mM for 2-oxo-pentanoate. K_m and V_{max} values were determined by fitting the kinetic data with GraphPad Prism 4.0 (GraphPad Software, Inc., La Jolla, CA) by using a nonlinear regression of the Hill equation.

The decarboxylase activity with phenylpyruvate, ketoisovalerate, ketomethylvalerate, ketoisocaproate, or methylthio-2-oxo-butanoate as the substrate was measured by monitoring NAD^+ reduction in the presence of excess aldehyde dehydrogenase from yeast, using a Tecan GENios Pro (Tecan) (57). The reaction mixture contained, in a total volume of 300 μl, 100 mM KH_2PO_4-K_2HPO_4 buffer (pH 7.0), 2 mM NAD^+, 5 mM $MgCl_2$, 15 mM pyrazole, 0.2 mM thiamine diphosphate, and 1.75 U of aldehyde dehydrogenase from yeast (Sigma-Aldrich) (dissolved in 1 mM dithiothreitol). The reaction was started by the addition of the substrate at concentrations ranging from 0.025 mM to 10 mM for phenylpyruvate and from 0.25 mM to 30 mM for the other substrates. Reaction rates were linearly proportional to the amount of cell extract added. For determinations of K_m and V_{max} values, the kinetic data were fitted with GraphPad Prism 4.0 (GraphPad Software, Inc.) by using a nonlinear regression of the Michaelis-Menten equation.

Determination of protein concentrations. Protein concentrations in cell extracts were determined by the method of Lowry et al. (36). Bovine serum albumin (Sigma-Aldrich) was used as a standard.

RESULTS

Expression of single decarboxylase genes in a decarboxylase-negative *S. cerevisiae* strain. To analyze the substrate specificity of TPP-dependent decarboxylases in *S. cerevisiae*, untagged versions of individual decarboxylase genes were expressed from strong constitutive promoters (*TDH3* and *PGK1*) in a host strain devoid of all chromosomal 2ODC genes (CEN.PK711-7C [*pdc1*Δ *pdc5*Δ *pdc6*Δ *aro10*Δ *thi3*Δ]) (Fig. 1). However, we verified that the *TDH3* and *PGK1* promoters showed similar transcriptional activities (varying by less than 25%) in ethanol-limited chemostat cultures (9, 32), fermentation conditions which were used in this study. In comparison with the expression of tagged versions of the encoded proteins in *Escherichia coli* (31), this approach prevents possible artifacts resulting from the presence of a fused tag. Moreover, by expressing the decarboxylases in *S. cerevisiae*, potential yeast-specific posttranslational modifications that might affect substrate specificity and protein stability are retained.

Cell extracts of quintuple deletion strain CEN.PK711-7C did not show measurable 2ODC activity, confirming its suitability as a platform for the individual overexpression of the five genes of interest (*PDC1*, *PDC5*, *PDC6*, *ARO10*, and *THI3*). The absence of pyruvate decarboxylase activity rendered strain CEN.PK711-7C unable to grow on glucose (16). To avoid possible impacts of the cultivation conditions, all cultures used for the preparation of cell extracts, including those of strains expressing a functional *PDC* gene, were grown on ethanol as the sole carbon source.

Strain IMZ002, in which *ARO10* was constitutively expressed, showed no 2ODC activity in cell extracts when it was grown on ethanol with ammonium sulfate as the nitrogen source (data not shown). The activity of Aro10 was previously shown to depend on the nitrogen source used for growth (60). Therefore, strain IMZ002 was grown on phenylalanine rather than ammonium sulfate as the nitrogen source.

Cell extracts were prepared from duplicate ethanol-limited chemostat cultures of each strain, followed by an analysis of decarboxylation kinetics with eight substrates: pyruvate, 2-oxo-butanoate, 2-oxo-pentanoate, phenylpyruvate, 3-methyl-2-oxo-pentanoate (ketomethylvalerate [KMV]), 4-methyl-2-oxo-pentanoate (ketoisocaproate [KIC]), 3-methyl-2-oxo-butanoate (ketoisovalerate [KIV]), and 4-methylthio-2-oxobutanoate (MTOB).

Decarboxylation of C_4 and C_5 linear 2-oxo acids requires a functional pyruvate decarboxylase. Pyruvate and 2-oxo-butanoate (30, 38, 39, 47) are both naturally occurring and essential intermediates of yeast metabolic pathways. A third linear 2-oxo acid, 2-oxo-pentanoate, has not been reported to occur as an intermediate in yeast metabolism. The activities of Pdc1, Pdc5, Pdc6, Aro10, and Thi3 with these linear 2-oxo acids were tested by analyzing the decarboxylation of different substrates by cell extracts of the corresponding "single-decarboxylase" strains. *S. cerevisiae* strains that expressed only *ARO10* or *THI3* did not show detectable decarboxylase activities with any of the three linear 2-oxo acids (Table 4). In contrast, high decarboxylase activities with each of these three substrates were observed for strains expressing *PDC1*, *PDC5*, or *PDC6* (Table 4). Except for an almost 2-fold-higher K_m of Pdc5 for pyruvate, the K_m and V_{max} values of the three pyruvate decarboxylase isoenzymes for these three linear substrates differed by less than 20% (Table 4). The V_{max} values of all three PDC isoenzymes were systematically lower with 2-oxo-butanoate and 2-oxo-pentanoate than with pyruvate (Table 4). However, the K_m values were lower for 2-oxo-butanoate and 2-oxo-pentanoate than for pyruvate. Pyruvate decarboxylase is known to exhibit cooperativity with its substrate pyruvate (26). Cooperativity was also found for the decarboxylation of 2-oxo-butanoate and 2-oxo-pentanoate by the three pyruvate decarboxylase isoenzymes, as reflected by their Hill coefficients for these substrates (Table 4).

Pyruvate decarboxylase is essential for *in vivo* production of *n*-propanol and *n*-butanol. 2-Oxo-butanoate and 2-oxo-pentanoate can be formed by the transamination of threonine and of the nonproteinogenic amino acid norvaline, respectively. To investigate the role of pyruvate decarboxylases in the *in vivo* decarboxylation of 2-oxo-butanoate and 2-oxo-pentanoate, the growth of a *pdc1*Δ *pdc5*Δ *pdc6*Δ strain was compared with that of a reference strain and with that of an *aro10*Δ strain during growth on ammonium, threonine, or norvaline as the sole nitrogen source. Since growth on ethanol was extremely slow when one of these amino acids was used as a nitrogen source (data not shown), *S. cerevisiae* strain IMI078 (*pdc1*Δ *pdc5*Δ *pdc6*Δ $mth1^{\Delta T}$) was used. This strain carries a 225-bp internal deletion in the *MTH1* gene, which eliminates the glucose sensitivity of Pdc^- strains and

TABLE 4 Specific decarboxylase activities for linear-chain 2-oxo acids in cell extracts of *S. cerevisiae* strains expressing individual genes with sequence similarity to genes encoding TPP-dependent decarboxylases[a]

Substrate	Strain (gene regulation)[b]	Mean K_m (mM) ± SD	Mean V_{max} (μmol · mg of protein^{-1} · min^{-1}) ± SD	Mean Hill coefficient ± SD	V_{max}/K_m ratio
Pyruvate	IMZ030 (*PDC1* ↑)	2.8 ± 0.1	1.5 ± 0.1	1.9 ± 0.1	0.52
	IMZ024 (*PDC5* ↑)	5.1 ± 0.1	1.3 ± 0.1	2.3 ± 0.2	0.26
	IMZ031 (*PDC6* ↑)	2.9 ± 0.1	1.5 ± 0.1	2.2 ± 0.1	0.52
	IMZ002 (*ARO10* ↑)	NA	BD	NA	NA
	IMZ025 (*THI3* ↑)	NA	BD	NA	NA
2-Oxo-butanoate	IMZ030 (*PDC1* ↑)	1.0 ± 0.1	0.5 ± 0.1	2.5 ± 0.2	0.52
	IMZ024 (*PDC5* ↑)	1.2 ± 0.1	0.4 ± 0.1	3.2 ± 0.5	0.37
	IMZ031 (*PDC6* ↑)	1.0 ± 0.1	0.5 ± 0.1	4.3 ± 0.9	0.44
	IMZ002 (*ARO10* ↑)	NA	BD	NA	
	IMZ025 (*THI3* ↑)	NA	BD	NA	
2-Oxo-pentanoate	IMZ030 (*PDC1* ↑)	1.5 ± 0.1	0.4 ± 0.1	2.7 ± 0.3	0.29
	IMZ024 (*PDC5* ↑)	1.5 ± 0.1	0.4 ± 0.1	2.4 ± 0.3	0.30
	IMZ031 (*PDC6* ↑)	1.6 ± 0.1	0.4 ± 0.1	2.7 ± 0.3	0.23
	IMZ002 (*ARO10* ↑)	NA	BD	NA	NA
	IMZ025 (*THI3* ↑)	NA	BD	NA	NA

[a] The data and mean deviations result from enzymatic assays performed with various substrate concentrations with cell extracts derived from duplicate carbon-limited chemostat cultivations. V_{max} and K_m values were calculated from single-component Michaelis-Menten fits. The Hill coefficients were calculated from the Hill equation; a hill coefficient of >1 denotes a positively cooperative reaction, which may be interpreted to mean that "once one ligand molecule is bound to the enzyme, its affinity for other ligand molecules increases." NA, not applicable; BD, below the detection limit with a V_{max} value of <0.02 μmol · mg of protein^{-1} · min^{-1}.
[b] ↑ denotes overexpression of the TPP-dependent 2-oxo acid decarboxylase.

thereby enables fast growth in batch cultures on glucose-ethanol mixtures (17; B. Oud, personal communication).

Reference strain CEN.PK113-7D produced up to 0.7 mM *n*-propanol during growth in shake flask cultures on a glucose-ethanol mixture with threonine as the nitrogen source (Fig. 2). Consistent with the absence of 2-oxo-butanoate decarboxylase activity in cell extracts of a single-decarboxylase strain expressing only Aro10 (Table 4), a similar concentration of *n*-propanol was found in cultures of *aro10*Δ strain CEN.PK555-4A. In contrast, no *n*-propanol was observed for cultures of strain IMI078 (*pdc1*Δ *pdc5*Δ *pdc6*Δ *mth1*$^{\Delta T}$), neither with ammonium as the nitrogen source nor with threonine or norvaline. Moreover, the growth rate of strain IMI078 on threonine was severely affected (Fig. 2). While *n*-propanol production by pyruvate decarboxylase-positive strains was increased by only 2.8-fold in cultures grown with threonine as the nitrogen source relative to the level of production in ammonium-grown cells (Fig. 2), the concentration of amyl alcohol (2-methyl-butanol) reached values above 6 mM in strains CEN.PK113-7D and CEN.PK555-4A, which represents a 43-fold increase when grown in the presence of threonine compared to the concentration when strains were grown in the presence of ammonium. These results suggest that under the experimental conditions used, the catabolism of the carbon skeleton of threonine proceeds mainly via the isoleucine biosynthetic pathway rather than via the direct decarboxylation of 2-oxo-butanoate in the Ehrlich pathway. However, the excess amount of 3-methyl-2-oxo-pentanoate (isoleucine 2-oxo-acid precursor) is then subsequently processed through the Ehrlich pathway, yielding amyl alcohol. We also cannot exclude that 2-oxo-butanoate proceeds via additional pathways (e.g., via the *GLY1*-encoded threonine aldolase [55]).

In cultures of reference strain CEN.PK113-7D grown with norvaline as the nitrogen source, *n*-butanol was detected at concentrations of up to 2.8 mM (210 mg · liter^{-1}). However, *n*-butanol was not detected in cultures grown with ammonium or threonine as the nitrogen source (Fig. 2). Similar results were obtained with a strain in which *ARO10* was deleted. In contrast, the deletion of the three *PDC* genes in strain IMI078 not only eliminated *n*-butanol production but also abolished growth on norvaline as the nitrogen source (Fig. 2).

Aro10p has superior kinetic properties for branched-chain, aromatic, and sulfur-containing 2-oxo acids. Branched-chain (KMV, KIC, and KIV), sulfur-containing (MTOB), and aromatic (phenylpyruvate) 2-oxo acids are derived from the transamination of amino acids (isoleucine, leucine, valine, methionine, and phenylalanine, respectively). Phenylpyruvate was previously shown to be efficiently decarboxylated by Aro10 (60, 61). Nevertheless, an *aro10*Δ strain still produced phenylethanol during growth on phenylalanine as the sole nitrogen source, indicating the presence of at least one other enzyme capable of decarboxylating phenylpyruvate (60, 61). As previously suggested (61), Pdc5 could efficiently act on phenylpyruvate, and its catalytic properties could be fitted by a Michaelis-Menten plot (Fig. 3). Pdc5 exhibited a lower V_{max} and a higher K_m than Aro10 (Fig. 3 and Table 5), thus confirming its role as an alternative phenylpyruvate decarboxylase. The decarboxylation kinetics for the remaining 2-oxo acids were analyzed at substrate concentrations ranging from 0.1 to 30 mM. Consistent with a previously reported suggestion that *THI3* does not encode a functional 2-oxo-acid decarboxylase (10), cell extracts of the "*THI3*-only" strain IMZ025 did not show detectable decarboxylase activity with any of the substrates tested (Table 5).

Aro10, which cannot decarboxylate pyruvate, exhibited clear Michaelis-Menten-type saturation kinetics for all other substrates tested (Table 5). In contrast, strains expressing either Pdc1, Pdc5, or Pdc6 as the sole decarboxylase showed a nonsaturated, linear increase in the level of enzyme activity with increasing concentrations of KMV, KIC, KIV, and MTOB (illustrated for KIC in Fig. 4), thereby indicating a K_m value above 20 mM. In order to compare

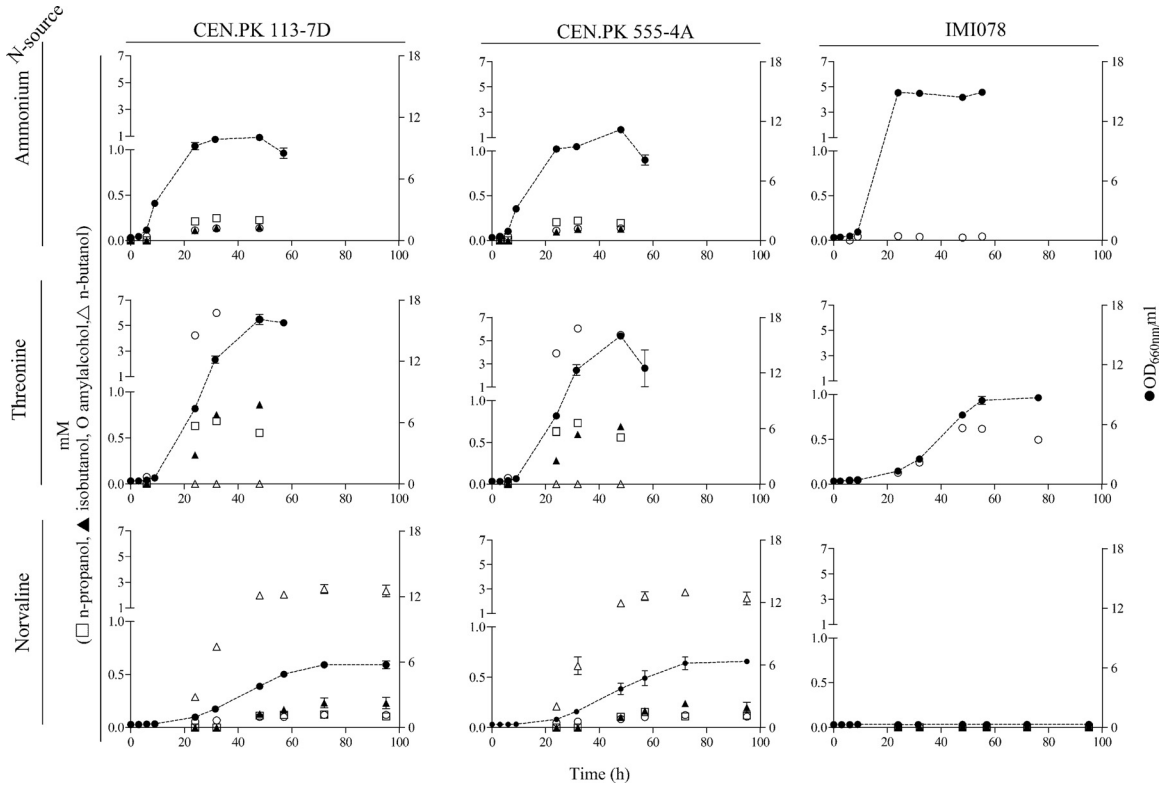

FIG 2 Growth and production of fusel alcohols in batch cultures of *S. cerevisiae* CEN.PK113-7D (reference), CEN.PK555-4A (*aro10*Δ), and IMI078 (*pdc1*Δ *pdc5*Δ *pdc6*Δ *mth1*$^{\Delta T}$). Strains were grown in shake flasks containing ammonium, threonine, or norvaline as the sole nitrogen source. Symbols: ●, biomass; ▲, isobutanol; □, *n*-propanol; ○, amyl alcohols; △, *n*-butanol. The optical density (OD) measurements as well as the concentrations of higher alcohols represent the average values and experimental deviations of data from duplicate cultures.

the activities of Aro10, Pdc1, Pdc5, and Pdc6, reaction rates with these 2-oxo acids were therefore analyzed at a fixed substrate concentration of 10 mM. Even at this comparatively high concentration, the decarboxylase activities of Pdc1, Pdc5, and Pdc6 were much lower than those of Aro10 (Table 5 and Fig. 4).

DISCUSSION

Previous biochemical studies of the substrate specificity of pyruvate decarboxylase purified from wild-type *S. cerevisiae* strains (33, 34, 51) likely analyzed mixtures of different pyruvate decarboxylase isoenzymes and, potentially, Aro10. Similarly, interpretations of data from previously reported substrate specificity studies based on *S. cerevisiae* mutants in which individual decarboxylase genes were inactivated (61) are complicated by the overlapping substrate specificities of the encoded enzymes. Compensatory regulatory mechanisms further complicate the interpretation of results obtained with deletion mutants. This problem is exemplified by the strong transcriptional upregulation of *PDC5* in *pdc1*Δ mutants (48) and in an *aro10*Δ strain grown in the presence of phenylalanine (60).

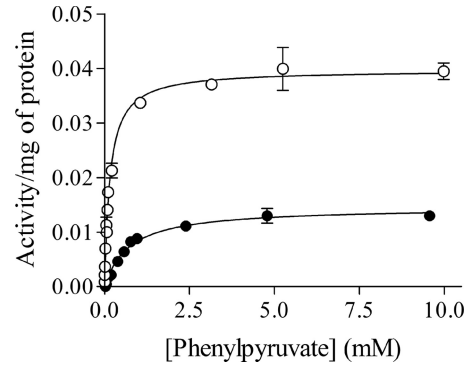

FIG 3 Kinetics of phenylpyruvate decarboxylase in cell extracts of *S. cerevisiae* strains IMZ002 (○) (*ARO10* ↑) and IMZ024 (●) (*PDC5* ↑). Enzyme activities were assayed for cell extracts of aerobic carbon-limited chemostat cultures. The data represent the averages ± standard deviations of the technical replicates ($n = 2$) of results from two independent chemostat cultures. ↑ denotes overexpression of the TPP-dependent 2-oxo acid decarboxylase.

By individually expressing decarboxylase genes in a "decarboxylase-negative" *S. cerevisiae* strain, the present study enabled a first systematic analysis of the substrate specificity of the TPP-dependent decarboxylases in *S. cerevisiae*. The main conclusions from *in vitro* enzyme assays with cell extracts were that (i) the substrate specificities of the three pyruvate decarboxylase isoenzymes do not differ significantly, with the exception of a higher level activity of Pdc5 with phenylpyruvate and a higher K_m of this isoenzyme for pyruvate; (ii) no evidence was found for a decarboxylating activity of Thi3, suggesting that previous reports (10, 11) of the impact of *thi3* mutations on fusel alcohol production reflect a regulatory role of Thi3 in thiamine biosynthesis rather than a catalytic activity; (iii) the linear 2-oxo acids pyruvate, 2-oxo-butanoate, and 2-oxo-pentanoate are decarboxylated only by the three pyruvate decarboxylase isoenzymes and not by Aro10p (Fig. 5); and (iv) Aro10p has a superior affinity for branched-chain, aromatic, and

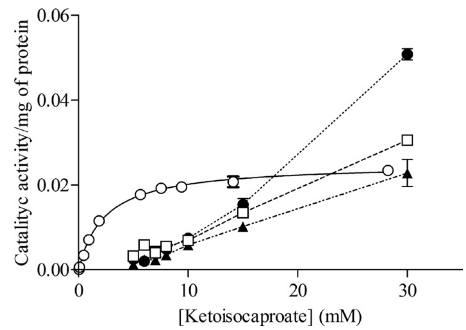

FIG 4 Kinetic of ketoisocaproate activity measured in cell extracts of *S. cerevisiae* strains IMZ002 (○) (*ARO10* ↑), IMZ024 (●) (*PDC5* ↑), IMZ030 (▲) (*PDC6* ↑), and IMZ031 (□) (*PDC1* ↑). Enzyme activities were assayed for cell extracts of aerobic carbon-limited chemostat cultures. The data represent the averages ± standard deviations of the technical replicates ($n = 2$) of results from two independent chemostat cultures. ↑ denotes overexpression of the TPP-dependent 2-oxo acid decarboxylase.

sulfur-containing 2-oxo acids in comparison with the affinities of the three pyruvate decarboxylase isoenzymes (Table 5 and Fig. 5).

Although the transcriptional regulation of *ARO10* has not been exhaustively investigated, its transcription is known to be strongly induced during growth with several amino acids as the nitrogen source (5, 28). The transcriptional induction of *ARO10* is mediated by the positive regulator Aro80p, whose synthesis is subject to nitrogen catabolite repression (28). Since the fermentation of alcoholic beverages generally involves the utilization of relatively diluted amino acid mixtures, *ARO10* is expressed in such processes, as confirmed by transcriptome analyses of beer (29). Together with its kinetic properties, this indicates that Aro10 is likely to be the main contributor to the major flavor-related decarboxylation reactions during the fermentation of alcoholic beverages with *S. cerevisiae*. The K_m values of Aro10 found in the present study corresponded well with data obtained previously with a tagged version of this protein expressed in *E. coli* (31), with the notable exception of a ca. 10-fold-higher K_m for MTOB in our study. This difference may be due to the use of an *ARO10* gene from a different *S. cerevisiae* strain, the presence of a histidine tag, or different posttranslational modifications of Aro10p in *E. coli.*

In vivo experiments confirmed that the production of *n*-propanol by *S. cerevisiae* depends on the activity of pyruvate decarboxylase (Fig. 5). Even when threonine, whose deamination by threonine dehydratase or transamination yields 2-oxo-butanoate (44), was absent from growth media, pyruvate decarboxylase-expressing strains produced significant concentrations of *n*-propanol. Apparently, consistent with their high affinity (V_{max}/K_m) for 2-oxo-butanoate found in cell extracts, pyruvate decarboxylase can compete efficiently for this substrate *in vivo* with isoleucine biosynthesis. This high affinity, combined with the high-level expression of pyruvate decarboxylase in anaerobic, fermentative yeast cultures (54), provides an adequate explanation for the occurrence of low concentrations of *n*-propanol in a wide range of yeast-based alcoholic beverages (i.e., wine, cider, and brandy [43, 50, 63]). Pyruvate decarboxylase is also likely to be responsible for the production of propanoate by aerobic *S. cerevisiae* cultures (35). The methylcitrate pathway for propanoate metabolism in *S. cerevisiae* (37, 45) can, in principle, act as a salvage pathway to recover carbon that is "lost" via 2-oxo-butanoate decarboxylation.

TABLE 5 Specific decarboxylase activities for aromatic, branched-chain, and sulfur-containing 2-oxo acids in cell extracts of *S. cerevisiae* strains expressing individual genes with sequence similarity to genes encoding TPP-dependent decarboxylases[a]

Strain (gene regulation)[b]	Phenylpyruvate		Ketoisovalerate		Ketoisocaproate		Ketomethylvalerate		4-Methylthio-2-oxo-butanoate	
	Mean V_{max} (μmol · mg of protein^{-1} · min^{-1}) ± SD	Mean K_m (mM) ± SD	Mean V_{max} (μmol · mg of protein^{-1} · min^{-1}) ± SD	Mean K_m (mM) ± SD	Mean V_{max} (μmol · mg of protein^{-1} · min^{-1}) ± SD	Mean K_m (mM) ± SD	Mean V_{max} (μmol · mg of protein^{-1} · min^{-1}) ± SD	Mean K_m (mM) ± SD	Mean V_{max} (μmol · mg of protein^{-1} · min^{-1}) ± SD	Mean K_m (mM) ± SD
IMZ030 (*PDC1* ↑)	BD	NA	38 ± 5	>20	15 ± 3	>20	9 ± 4	>20	41 ± 9	>20
IMZ024 (*PDC5* ↑)	68 ± 9	0.67 ± 0.07	46 ± 16	>20	19 ± 4	>20	8 ± 1	>20	54 ± 2	>20
IMZ031 (*PDC6* ↑)	BD	NAa	31 ± 5	>20	19 ± 3	>20	10 ± 1	>20	51 ± 7	>20
IMZ002 (*ARO10* ↑)	201 ± 29	0.14 ± 0.01	103 ± 33	12 ± 2	103 ± 11	2.1 ± 0.2	103 ± 10	4.7 ± 0.5	85 ± 4	5.36 ± 0.7
IMZ025 (*THI3* ↑)	BD	NA	BD	NA	BD	NA	BD	NA	BD	NA
IMZ001 (Pdc^{-})	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD

[a] Shown are data and mean results from single-component Michaelis-Menten fits of the averages of technical replicates ($n = 2$) of results from two independent chemostat cultures. NA, not applicable; BD, below the detection limit with a V_{max} of <8 nmol · mg of protein^{-1} · min^{-1}.
[b] ↑ denotes overexpression of the TPP-dependent 2-oxo acid decarboxylase.

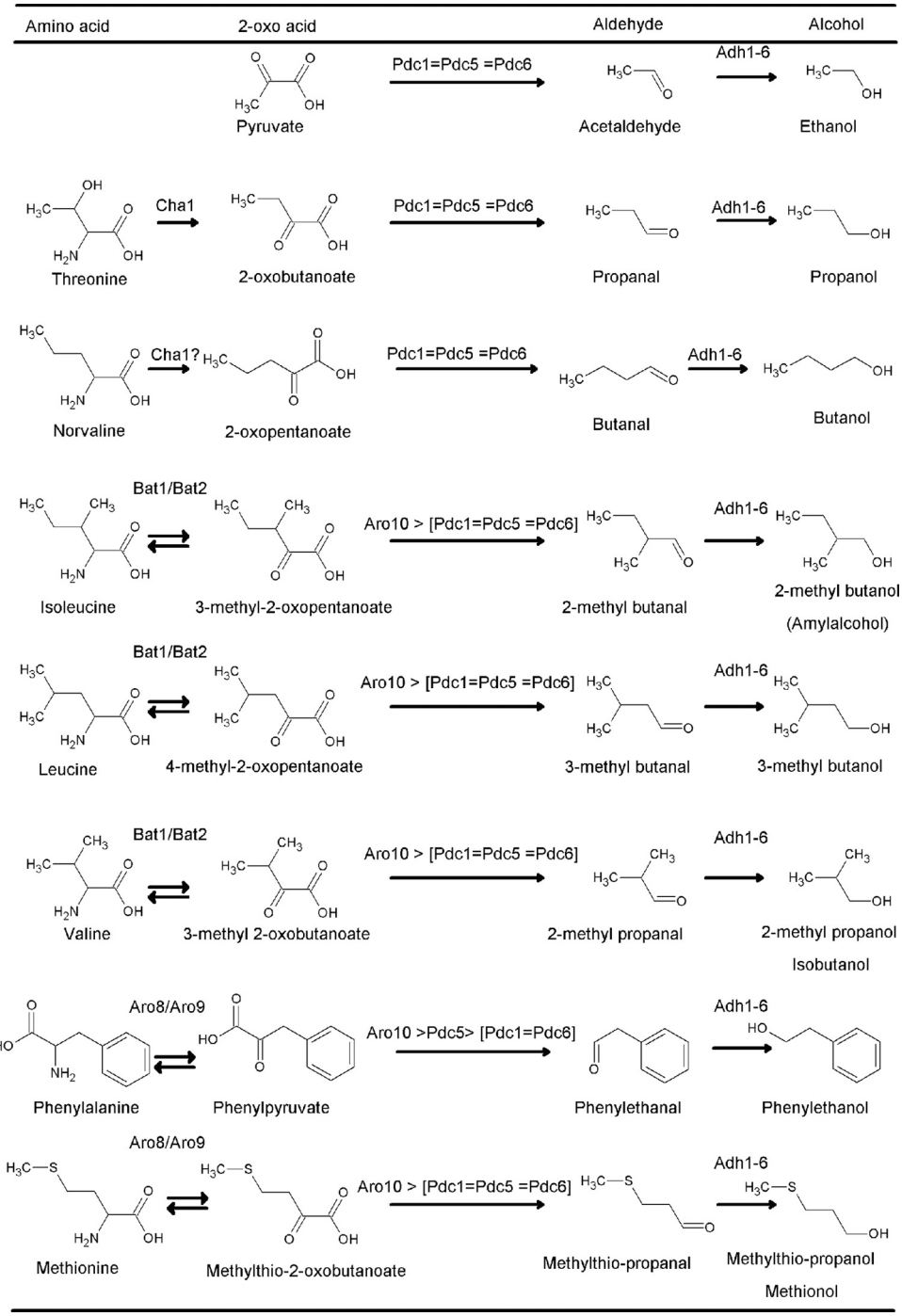

FIG 5 Overview of the role of the five 2ODCs in the formation of higher alcohols.

Unlike 2-oxo-butanoate, 2-oxo-pentanoate is not an essential intermediate in yeast central carbon metabolism. Consistently, we observed the production of *n*-butanol only in pyruvate decarboxylase-expressing cultures grown with norvaline, the amino acid precursor of this fusel alcohol. In bacteria, 2-oxo-pentanoate can be formed as a side product of reactions in branched-chain-amino-acid synthesis and can subsequently be aminated to norvaline (6). Norvaline synthesis in *E. coli* depends strongly on the cultivation conditions (6), and an early study showed *n*-butanol production in *S. cerevisiae* strains mutated in branched-chain-amino-acid metabolism (21, 27). This indicates that in *S. cerevisiae*, a native pathway for 2-oxo-butanoate synthesis operates in specific genetic backgrounds and, conceivably, under special environmental conditions. By analogy with previous studies of norvaline production in *E. coli* (6), such a pathway could encompass the carbon chain elongation of 2-oxo-butanoate by the leucine biosynthesis enzymes Leu4/9, Leu2, and Leu1. Alternatively, the frequent occurrence of low con-

centrations of *n*-butanol in fermented beverages (50, 63) may reflect the pyruvate decarboxylase-mediated decarboxylation of plant-derived 2-oxo-pentanoate by *S. cerevisiae*. Extensive metabolic engineering of *S. cerevisiae* for the expression of a *Clostridium* *n*-butanol pathway (49) resulted in *n*-butanol titers that were 2 orders of magnitude lower than those observed for norvaline-grown batch cultures of a wild-type *S. cerevisiae* strain (Fig. 2). The engineering of an *n*-butanol pathway involving 2-oxo-pentanoate as an intermediate may offer an interesting alternative to the introduction of a *Clostridium*-type pathway.

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