

An Investigation of the Metabolism of Isoleucine to Active Amyl Alcohol in *Saccharomyces cerevisiae**

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The metabolism of isoleucine to active amyl alcohol (2-methylbutanol) in yeast was examined by the use of ^{13}C nuclear magnetic resonance spectroscopy, combined gas chromatography-mass spectrometry, and a variety of mutants. From the identified metabolites a number of routes between isoleucine and active amyl alcohol seemed possible. All involved the initial decarboxylation of isoleucine to α -keto- β -methylvalerate. The first, via branched chain α -ketoacid dehydrogenase to α -methylbutyryl-CoA, was eliminated because abolition of branched-chain α -ketoacid dehydrogenase in an *lpd1* disruption mutant did not prevent the formation of active amyl alcohol. However, the *lpd1* mutant still produced large amounts of α -methylbutyrate which initially seemed contradictory because it had been assumed that α -methylbutyrate was derived from α -methylbutyryl-CoA via acyl-CoA hydrolase. Subsequently it was observed that α -methylbutyrate arises from the non-enzymic oxidation of α -methylbutyraldehyde (the immediate decarboxylation product of α -keto- β -methylvalerate). Mutant studies showed that one of the decarboxylases encoded by *PDC1*, *PDC5*, *PDC6*, *YDL080c*, or *YDR380w* must be present to allow yeast to utilize α -keto- β -methylvalerate. Apparently, any one of this family of decarboxylases is sufficient to allow the catabolism of isoleucine to active amyl alcohol. This is the first demonstration of a role for the gene product of *YDR380w*, and it also shows that the decarboxylation steps for each α -keto acid in the catabolic pathways of leucine, valine, and isoleucine are accomplished in subtly different ways. In leucine catabolism, the enzyme encoded by *YDL080c* is solely responsible for the decarboxylation of α -ketoisocaproate, whereas in valine catabolism any one of the isozymes of pyruvate decarboxylase will decarboxylate α -ketoisovalerate.

leucine and valine in *Saccharomyces cerevisiae* to the end products isoamyl alcohol and isobutyl alcohol, respectively (1, 2). Both amino acids are first deaminated yielding α -ketoisocaproate from leucine and α -ketoisovalerate from valine. Subsequently, the α -keto acids are decarboxylated by different enzymes as follows: α -ketoisocaproate is decarboxylated by α -ketoisocaproate decarboxylase (the product of the open reading frame *YDL080c* for which we suggested the gene name “*KIDI*” (1)), whereas any one of the isozymes of pyruvate decarboxylase (*PDC1*, *PDC5*, or *PDC6* gene products) will decarboxylate α -ketoisovalerate (2). Three major questions about the catabolism of the branched-chain amino acids in yeast remain unanswered. First, there has been no systematic examination of the route(s) of isoleucine catabolism. Notions first proposed over 90 years ago (3) and now known as the “Ehrlich pathway” are still assumed to be correct. Second, the enzyme(s) involved in the subsequent metabolism of the putative aldehydes to the fusel alcohol end products are also unknown. These are presumed to be alcohol dehydrogenases. It has been suggested that the ethanol dehydrogenases encoded by *ADH1* and *ADH2* that are involved in central carbon metabolism are responsible (4), but the evidence is not fully convincing. Furthermore, since that suggestion was made, two more ethanol dehydrogenases (encoded by *ADH3* and *ADH4*) (5, 6) and six other putative alcohol dehydrogenases (*AAD3*, *AAD4*, *AAD6*, *AAD10*, *AAD14*, *AAD15* and *AAD16*) (7, 8) have been discovered. Third, despite the fact that branched-chain α -ketoacid dehydrogenase has been purified from *S. cerevisiae* and its kinetic properties characterized (9, 10), there is no known physiological role for this activity. In an attempt to solve the first of these questions we started to determine the metabolic pathway(s) used in the catabolism of isoleucine to active amyl alcohol (2-methylbutanol).

EXPERIMENTAL PROCEDURES

Strains, Media, and Cultural Conditions—The strains used are shown in Table I. Standard genetic techniques (11, 12) were used in all cases. Strain 53.1.4 was constructed by mating strain MML22 to JRD719 and sporulation of the resultant diploid. Since both parents in this cross carried *ura3* mutations, haploid progeny segregating the *lpd1::URA3* mutation were recognized by the ability to grow on glucose minimal medium lacking uracil and the inability to grow on any media in which glycerol was the carbon source (10, 13–14). Enzyme assays showed that strain 53.1.4 lacked pyruvate dehydrogenase, α -ketoglutarate dehydrogenase, and branched-chain α -ketoacid dehydrogenase as a consequence of the *lpd1::URA3* mutation. Strain 55.2.1 was constructed by mating Y14216 to JRD329 and sporulation of the resultant diploid. Segregation of the *ydr380w::KANMX4* allele was followed by the ability to grow in the presence of the antibiotic G418 (geneticin at 200 $\mu\text{g}/\text{ml}$). Strain 56.9.2 was constructed by crossing Y14216 with JRD815–6.1. In this cross the genotypes of the progeny were established by the following four criteria. First, strains that carried both the *ydr380w::KANMX4* and *hdl080c::KANMX4* disruption alleles were identified in asci which segregated two G418-resistant and two G418-sensitive spore progeny. Second, those strains that were also *pdcl pdc5*

The catabolism of the branched-chain amino acids leucine, valine, and isoleucine is entirely different in yeasts from that found in other eukaryotes. In most eukaryotes the carbon skeletons derived from these amino acids enter the tricarboxylic acid cycle, whereas in yeasts the end products are the so-called “fusel” alcohols. We have previously used a combination of ^{13}C NMR spectroscopy, gas chromatography-mass spectrometry (GC-MS),¹ and selected mutants to examine the catabolism of

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¹The abbreviation used is: GC-MS, gas chromatography-mass spectrometry.

TABLE I
Yeast strains used

Strain	Complete genotype	Source or Ref.
IWD72	<i>MATα</i>	9
JRD719	<i>MATα ura3 trp1</i>	2
YSH5.127.-17C	<i>MATα leu2 ura3 trp1 pdc1Δ::LEU2 pdc5Δ::URA3 pdc6Δ::TRP1 GAL</i>	S. Hohmann ^a
JRD815-6.1	<i>MATα leu2 ura3 trp1 pdc1Δ::LEU2 pdc5Δ::URA3 pdc6Δ::TRP1 ydl080c::KANMX4</i>	1
53.1.4	<i>MATα ura3 lys2 met8 lpd1::URA3</i>	This work
MML22	<i>MATα leu2 ura3 his3 ade2 lys2 met8 gal80::HIS3 lpd1::URA3</i>	13
55.2.1	<i>MATα leu2 ade8 ydr380w::KANMX4</i>	This work
56.9.2	<i>MATα pdc1Δ::LEU2 pdc5Δ::URA3 pdc6Δ::TRP1 ydl080c::KANMX4 ydr380w::KANMX4</i>	This work
50.2.4	<i>MATα leu2 trp1 ydl080c::KANMX4</i>	This work
Y14216	<i>MATα leu2 ura3 his3 lys2 ydr380w::KANMX4</i>	EUROSCARF ^b
JRD329	<i>MATα ade8</i>	J. R. Dickinson
51.1.3	<i>MATα ura3 ydl080c::KANMX4</i>	J. R. Dickinson
57.2.13	<i>MATα ade8 leu2 ura3 ydl080c::KANMX4 ydr380w::KANMX4</i>	This work

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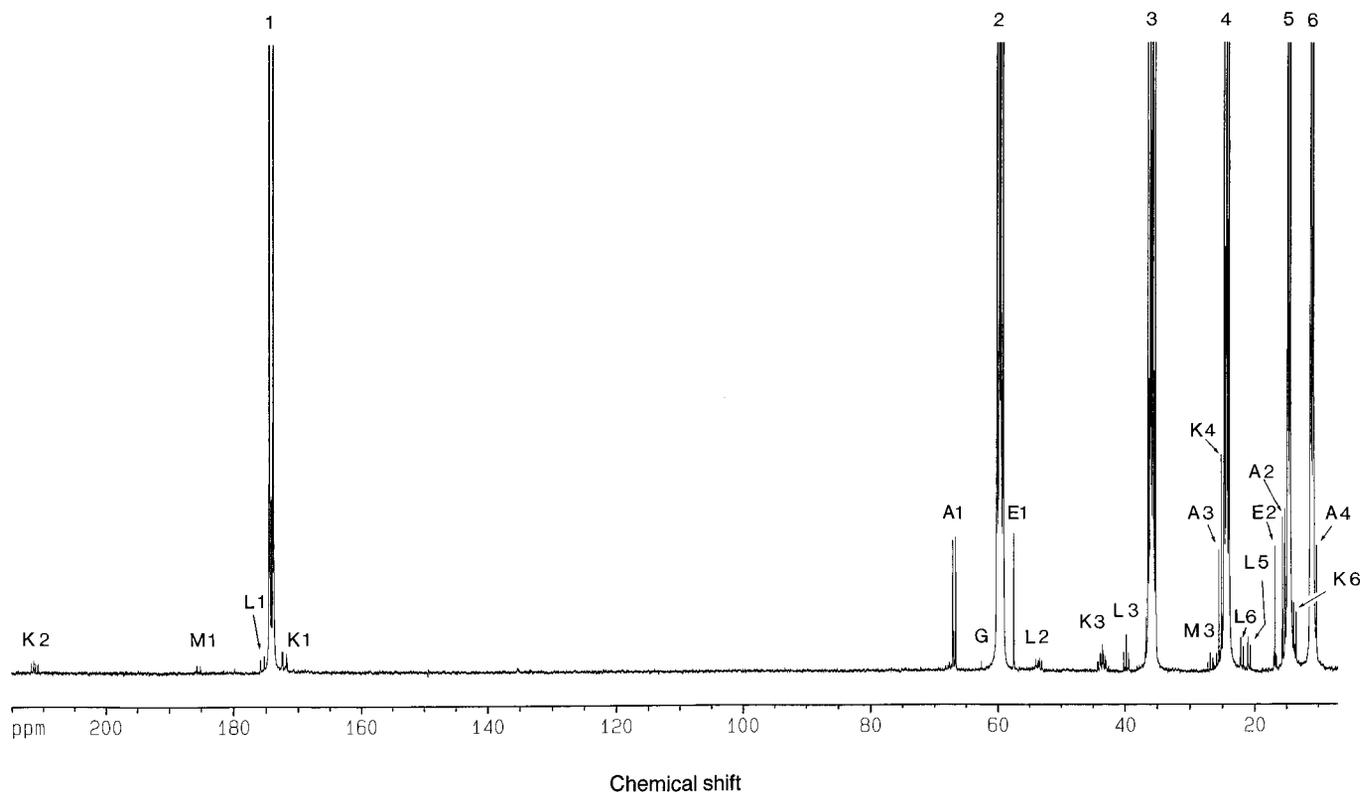


FIG. 1. The ¹³C NMR spectrum of a culture supernatant of the wild-type strain IWD72. Cells were cultured in glucose minimal medium with [U-¹³C]isoleucine as sole nitrogen source. The resonances marked 1-6 are C-1 to C-6 (respectively) of isoleucine. The other resonances indicate the corresponding carbon atoms (where evident) in each of the following compounds: *K*, α -keto- β -methylvalerate; *M*, α -methylbutyrate; *L*, leucine; *A*, active amyl alcohol; *G*, C-1 and C-3 of glycerol; *E*, ethanol.

pdc6 triple mutants were identified by their inability to grow on glucose-containing media. Third, strain 56.9.2 was backcrossed to Y14216 and 50.2.4. A *ydr380w::KANMX4 ydl080c::KANMX4* double mutant is expected to yield all G418-resistant progeny when backcrossed to a strain that carries either a *ydr380w::KANMX4* mutation or a *ydl080c::KANMX4* mutation, whereas a single mutant (in either gene) will give 25% G418-sensitive spores when backcrossed to a mutant defective in the other gene. Strain 56.9.2 gave entirely G418-resistant progeny in both backcrosses. Finally, the *ydr380w::KANMX4* and *ydl080c::KANMX4* disruptions were verified by diagnostic polymerase chain reactions using specific primers that yielded products that were unique to each disruption allele. Strain 57.2.13 was constructed by crossing strains 55.2.1 and 51.1.3. Spore progeny that carried both the *ydr380w::KANMX4* and *ydl080c::KANMX4* disruption alleles were identified as described above.

Starter cultures were grown in a medium comprising 1% yeast extract, 2% Bactopeptone, and 2% carbon source. For studies of isoleucine catabolism, cells were grown in minimal medium containing (per liter) 1.67 g of Yeast Nitrogen Base (Difco), 20 g of isoleucine, and either 20

ml of ethanol or 20 g of glucose for the carbon source. Experiments involving ¹³C labeling used [U-¹³C]isoleucine (99.9 atom %) from Cambridge Isotope Laboratories (Cambridge, MA). Auxotrophic requirements were supplied as required at 20 μ g/ml. Liquid cultures were grown in conical flasks filled to 40% nominal capacity in a gyrorotatory shaker. Agar (2%) was used to solidify media. All cell cultures were at 30 °C.

NMR Analyses—¹³C NMR spectra were recorded and signals identified exactly as described previously (1). Chemical shifts (δ , ppm) are reported relative to external tetramethylsilane in C²HCl₃; addition of sodium trimethylsilylpropanesulfonate gave a methyl signal at -2.6 ppm under the conditions used here.

Determination of Active Amyl Alcohol and α -Methylbutyrate Levels—Active amyl alcohol and α -methylbutyrate levels were determined in culture filtrates by gas chromatography-mass spectrometry (GC-MS) on a 30-m (0.32 mm internal diameter) fused silica capillary column with a 0.25- μ m film of Supelcowax 10 (Supelco) in a Voyager GC-MS (ThermoQuest Finnigan, Manchester, UK). The injector temperature was 250 °C, and the samples were chromatographed for 2 min at 60 °C

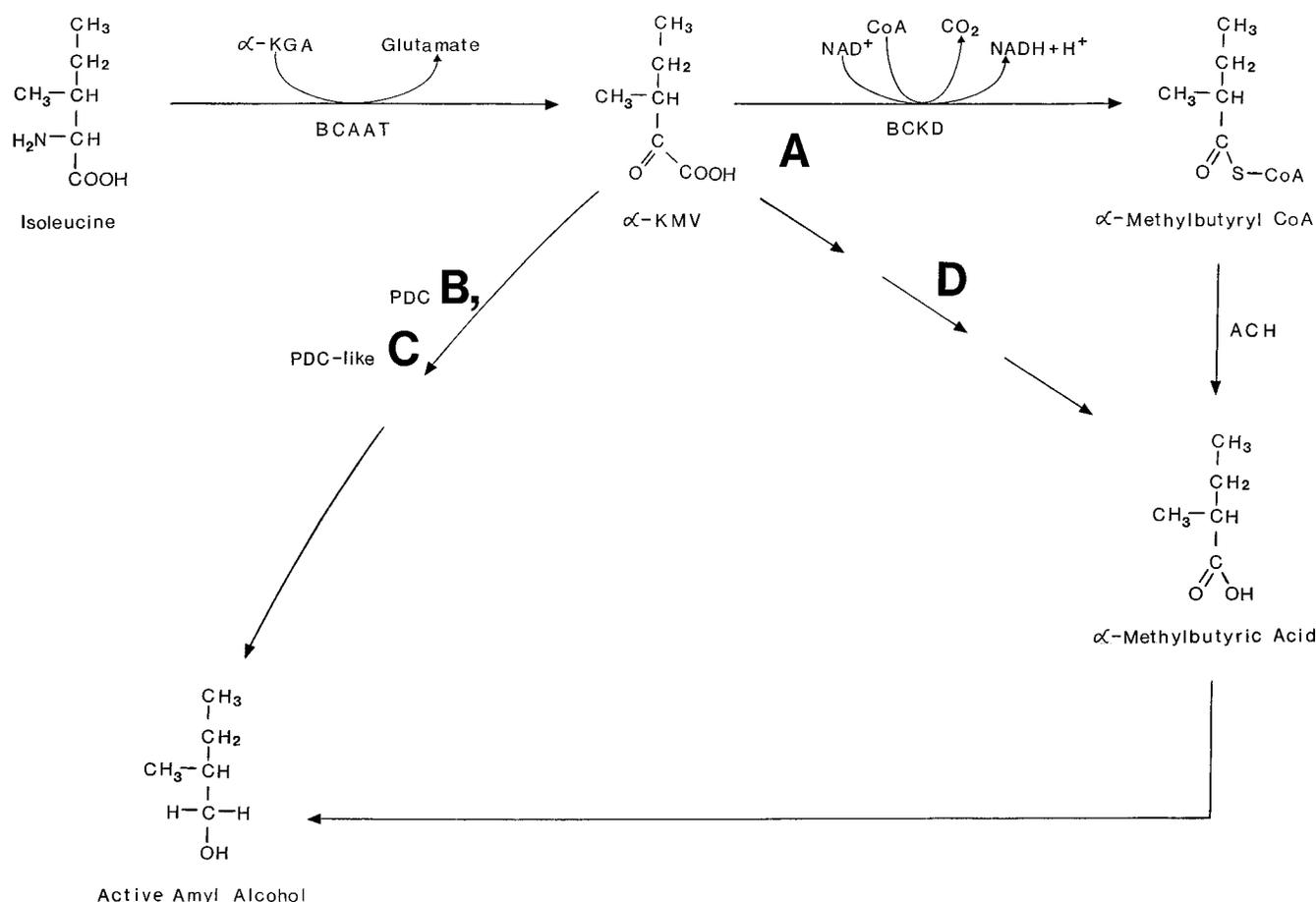


FIG. 2. Potential metabolic routes for the metabolism of isoleucine to active amyl alcohol. Enzyme activities that are already known are abbreviated. *BCAAT*, branched-chain amino acid amino transferase; *BCKD*, branched-chain α -ketoacid dehydrogenase; *ACH*, acyl-CoA hydrolase; *PDC*, pyruvate decarboxylase; α -*KMV*, α -keto- β -methylvalerate.

followed by a temperature increase of 10 °C/min to 200 °C which was then maintained for the remaining 4 min. Helium was used as the carrier gas at 30 kPa and a constant flow rate of 1.5 ml/min. Standard solutions of active amyl alcohol and α -methylbutyric acid gave linear calibrations over the range 0–1000 $\mu\text{g/ml}$.

RESULTS

Isoleucine Catabolism in a Wild-type Strain—Fig. 1 shows the ^{13}C NMR spectrum of a culture supernatant of a wild-type strain that had been cultured for 24 h in a minimal medium in which glucose was the carbon source and $[\text{U-}^{13}\text{C}]$ isoleucine the sole nitrogen source. The use of uniformly labeled isoleucine helped in the assignment of the resonances in the NMR spectrum. This is because every resonance in the metabolites derived from isoleucine is a multiplet. Analysis of the multiplets and their couplings allows unambiguous identification. The isoleucine multiplets (not shown) all displayed an average of 4.4% singlet due to the presence of unlabeled (^{12}C) isoleucine in the substrate from the supplier. Of the remaining 95.6% ^{13}C -labeled amino acids approximately 0.6% was $[\text{U-}^{13}\text{C}]$ isoleucine. Thus, only 95% of the material fed to yeast was actually $[\text{U-}^{13}\text{C}]$ isoleucine, and thus the leucine observed in the spectrum in Fig. 1 was due to this contaminant and not due to biosynthesis from isoleucine. The three compounds that were ^{13}C -labeled at every position were α -keto- β -methylvalerate, α -methylbutyrate, and active amyl alcohol. Clearly, these were all derived from the $[\text{U-}^{13}\text{C}]$ isoleucine substrate.

No natural abundance resonances of glucose were observed which indicates that all of the glucose had been consumed. However, resonances of ethanol and glycerol derived from the glucose were evident. From the metabolites identified a num-

ber of routes between isoleucine and active amyl alcohol seemed possible (Fig. 2). All involve the initial decarboxylation of isoleucine to α -keto- β -methylvalerate. Route "A" envisages oxidative decarboxylation of α -keto- β -methylvalerate via branched chain α -ketoacid dehydrogenase to yield α -methylbutyryl-CoA. Next, α -methylbutyryl-CoA could be converted by acyl-CoA hydrolase to α -methylbutyrate, which could then be reduced to active amyl alcohol. Alternatively, α -keto- β -methylvalerate could be decarboxylated by one or more of the isozymes of pyruvate decarboxylase (route "B") or a pyruvate decarboxylase-like enzyme (route "C"). The final possibility was route "D" involving either the direct or indirect conversion of α -keto- β -methylvalerate to α -methylbutyrate.

Isoleucine Catabolism in a Mutant That Lacks Branched-chain α -Ketoacid Dehydrogenase—A variety of mutants was deployed to elucidate the pathway used for the catabolism of isoleucine to active amyl alcohol. Mutant strain 53.1.4 (*lpd1::URA3*) which lacks lipoamide dehydrogenase and, as a consequence, also lacks pyruvate dehydrogenase, α -ketoglutarate dehydrogenase, branched-chain α -ketoacid dehydrogenase, and glycine decarboxylase (13–16) grew on minimal medium in which isoleucine was the sole source of nitrogen and also produced active amyl alcohol. Strain 53.1.4 grew to only one-quarter the density of the wild-type strain IWD72 (Fig. 3 and Fig. 4) which is not unexpected considering the metabolic disadvantages suffered by the *lpd1* mutant relative to the wild type. However, two other facts were remarkable. First, the levels of active amyl alcohol on a *per cell* basis were similar in both strains (Table II) indicating that branched-chain α -ketoacid dehydrogenase cannot be required for the formation of

active amyl alcohol. Second, large amounts of α -methylbutyrate were formed by both strains (Fig. 3 and Fig. 4). These results were clearly contrary to the idea that α -methylbutyrate is formed from α -methylbutyryl-CoA because the *lpd1* mutant cannot form α -methylbutyryl-CoA.

Since branched-chain α -ketoacid dehydrogenase had been excluded, it seemed likely that some sort of decarboxylase activity was involved instead. The non-oxidative decarboxylation of α -keto- β -methylvalerate would initially yield α -methylbutyraldehyde. A series of time-elapsing ^{13}C NMR spectra revealed that this compound oxidized very rapidly to α -methylbutyrate in sterile glucose minimal medium containing 2% isoleucine when incubated at 30 °C with shaking (data not shown). The sterile growth medium contained no α -methylbutyrate initially, so the compound was not formed upon autoclaving, and none was formed when growth medium was incubated without the addition of α -methylbutyraldehyde.

α -Methylbutyraldehyde oxidized more slowly at pH 6.0 in 50

mM phosphate buffer containing 15% $^2\text{H}_2\text{O}$ in a stationary 5-mm NMR tube at 20 °C. Even so, after 4 days, approximately 75% had become converted to α -methylbutyrate. Hence, the formation of α -methylbutyrate seems to be due entirely to chemical, rather than biochemical processes.

Isoleucine Catabolism in Mutants Lacking Various Decarboxylases—Strain YSH5.127.-17C (*pdc1 pdc5 pdc6*) which is totally devoid of pyruvate decarboxylase (17) and strain JRD815-6.1 (*pdc1 pdc5 pdc6 ydl080c::KANMX4*) which lacks pyruvate decarboxylase and α -ketoisocaproate decarboxylase (1) grew on ethanol minimal medium in which isoleucine was the sole source of nitrogen and produced similar amounts of active amyl alcohol both to each other and also to the wild-type strain IWD72 (Table II). Apparently, some other decarboxylase must be involved in this pathway.

A search of the *Saccharomyces* Genome Data base revealed the existence of the open reading frame YDR380w on chromosome IV which encodes a protein of unknown function with homology to the isozymes of pyruvate decarboxylase encoded by *PDC1*, *PDC5*, and *PDC6* and the α -ketoisocaproate decarboxylase encoded by *YDL080c* which we have suggested should be

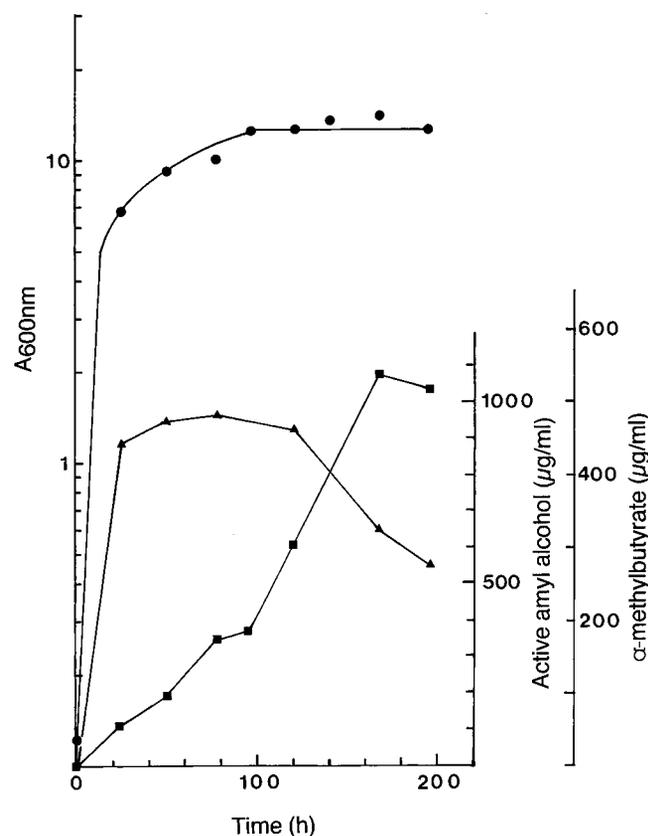


FIG. 3. Growth and active amyl alcohol formation by wild-type strain IWD72 in glucose minimal medium with isoleucine as sole nitrogen source. ●, $A_{600\text{nm}}$; ▲, active amyl alcohol in the medium ($\mu\text{g/ml}$); ■, α -methylbutyrate in the medium ($\mu\text{g/ml}$).

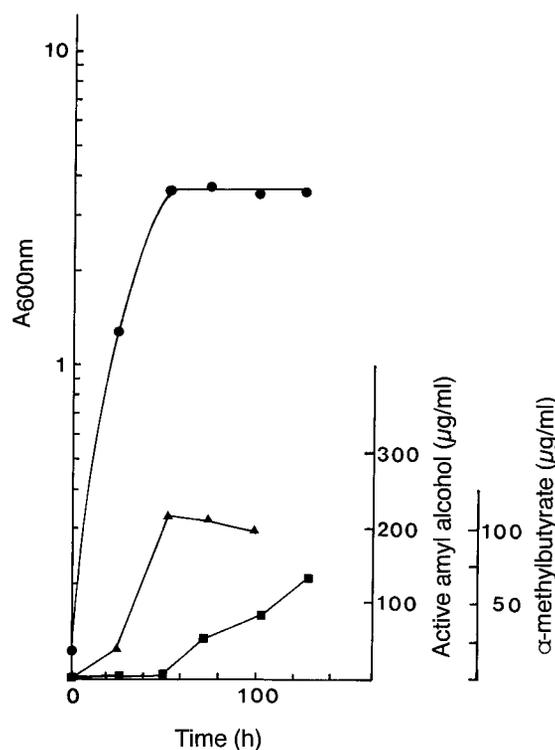


FIG. 4. Growth and active amyl alcohol formation by mutant strain 53.1.4 in glucose minimal medium with isoleucine as sole nitrogen source. ●, $A_{600\text{nm}}$; ▲, active amyl alcohol in the medium ($\mu\text{g/ml}$); ■, α -methylbutyrate in the medium ($\mu\text{g/ml}$).

TABLE II

Amount of active amyl alcohol produced by various strains growing in minimal medium with isoleucine as sole source of nitrogen

Active amyl alcohol content was determined in the deceleration phase when it was maximal. Values are the means of duplicate determinations. Differences between samples were <4%.

Strain	Relevant genotype	Carbon source	Active amyl alcohol	
			$\mu\text{g/ml}$	$\mu\text{g}/10^6$ cells
IWD72	Wild type	Glucose	958	9.68
IWD72	Wild type	Ethanol	290	3.63
YSH5.127.-17C	<i>pdc1 pdc5 pdc6</i>	Ethanol	396	7.07
JRD815-6.1	<i>pdc1 pdc5 pdc6 ydl080c::KanMX4</i>	Ethanol	145	3.26
53.1.4	<i>lpd1</i>	Glucose	213	5.88
55.2.1	<i>ydr380w::KANMX4</i>	Glucose	1024	11.36
56.9.2	<i>pdc1 pdc5 pdc6 ydl080c::KANMX4 ydr380w::KANMX4</i>	Ethanol	0	0
57.2.13	<i>ydl080c::KANMX4 ydr380w::KANMX4</i>	Glucose	526	7

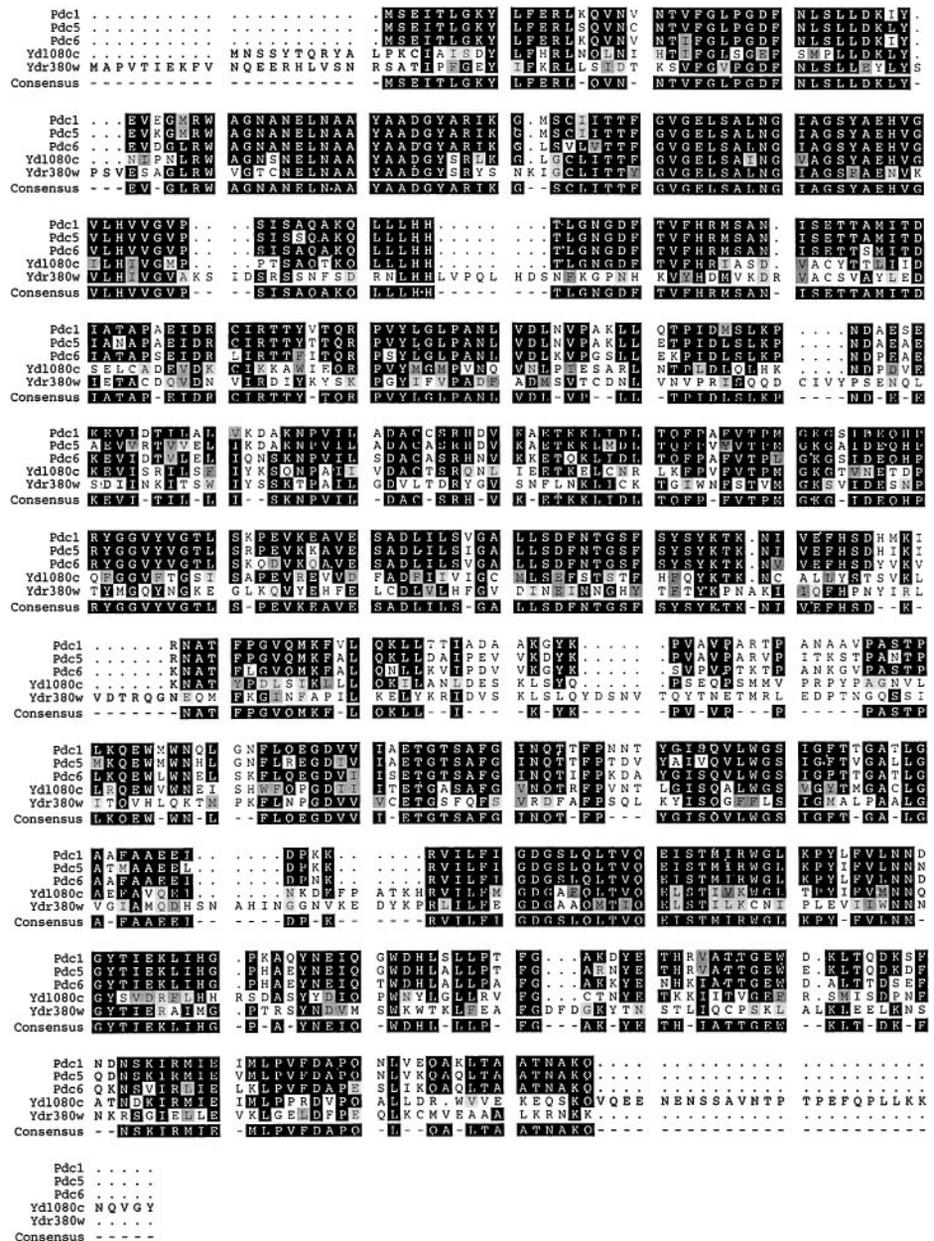


FIG. 5. Alignment of the predicted amino acid sequences of the three isozymes of pyruvate decarboxylase with the gene products of open reading frames YDL080c and YDR380w. The alignment was made using the PileUp program with a gap weight of 3.0 and a gap length weight of 0.1. The figure was produced using the PRETTYBOX program that displays identical residues in a black box and conservative substitutions in a gray box.

called *KID1* (1). Fig. 5 shows that the homologies between all five proteins are significant and extend over virtually the whole length of all of the proteins. YDL080c and YDR380w encode proteins with slightly extended N termini, and YDL080c also encodes a longer C terminus than all of the others. Apart from this, the decarboxylase and thiamine diphosphate binding site motifs are conserved. The protein encoded by YDR380w is 35% identical to Pdc1p.

Mutant strain 55.2.1 which has a deletion and disruption in YDR380w grew on isoleucine as sole nitrogen source and formed normal amounts of active amyl alcohol (Table II). Strain 56.9.2, which lacks all three isozymes of pyruvate decarboxylase as well as both of the decarboxylases encoded by YDL080c and YDR380w, also grew to high densities on ethanol minimal medium with isoleucine as sole source of nitrogen. However, this quintuple mutant did not produce active amyl alcohol (Table II). Strain 57.2.13 which is defective in both YDL080c and YDR380w grew well and produced normal amounts of active amyl alcohol (Table II). Taken together these results demonstrate that one of the decarboxylases encoded by *PDC1*, *PDC5*, *PDC6*, YDL080c, or YDR380w must be present to

allow yeast to utilize α -keto- β -methylvalerate. Apparently, any one of the decarboxylases is sufficient to allow the catabolism of isoleucine to active amyl alcohol. This is a degree of complexity not previously entertained for the Ehrlich pathway.

YDR380w has recently been shown to have Aro80p-dependent induction by tryptophan (18). With its obvious appearance as a decarboxylase, and regulation by tryptophan, it has been suggested that YDR380w could encode indolepyruvate decarboxylase (the enzyme that catalyzes the conversion of indole pyruvate to indole acetaldehyde in the tryptophan catabolic pathway) (18). With this in mind, strains carrying the *ydr380w::KANMX4* allele were examined for an inability to grow on minimal medium with 2% tryptophan as sole source of nitrogen. Although the original strain Y14216 from EURO-SCARF did not grow on tryptophan as sole nitrogen source, crosses to other strains showed this to be merely due to the FY1679 genetic background and not an inherent property of the *ydr380w* mutation. Of course, this simple experiment does not prove that YDR380w does not encode an indolepyruvate decarboxylase activity because there may be several decarboxylases that can decarboxylate indole pyruvate. However, strain

56.9.2 was able to grow on ethanol minimal medium with tryptophan as sole source of nitrogen, so if there is degeneracy of indolepyruvate decarboxylase, it must be accomplished by an enzyme other than Pdc1p, Pdc5p, Pdc6p, and Kid1p.

DISCUSSION

Analysis of the metabolism of [U-¹³C]isoleucine by the wild-type strain initially suggested four credible routes from α -keto- β -methylvalerate to active amyl alcohol (Fig. 2). Experiments using a range of different mutants led to the conclusion that any one of the decarboxylases encoded by *PDC1*, *PDC5*, *PDC6*, *YDL080c*, or *YDR380w* can catalyze the decarboxylation of α -keto- β -methylvalerate. Thus, the decarboxylation step for each α -keto acid in the catabolic pathways of leucine, valine, and isoleucine are accomplished in subtly different ways. In leucine catabolism, the enzyme encoded by *YDL080c* is solely responsible for the decarboxylation of α -ketoisocaproate (1) while in valine catabolism; any one of the isozymes of pyruvate decarboxylase will decarboxylate α -ketoisovalerate (2). Presumably, this degree of degeneracy allows the yeast cell to perform essential processes under a wide range of differing physiological conditions. However, this is currently mere speculation until the full spectrum of expression and regulation of all of these decarboxylases has been established.

Since *YDR380w* and *YDL080c* encode proteins capable of decarboxylating α -keto- β -methylvalerate, this means that the *YDL080c* gene product is involved in three biochemical pathways. In leucine catabolism it encodes α -ketoisocaproate decarboxylase activity which led us to suggest the gene name *KID1* (1). It has also been identified as "*THI3*" with a rather mysterious role in thiamine biosynthesis (19–20). Perhaps this gene has even more roles in metabolism that have not yet been discerned. Of course, if these roles are shared with other genes, they will only be discovered when *YDL080c* is deleted along with the other gene(s) involved. *YDR380w* is involved in isoleucine catabolism and possibly also tryptophan catabolism. There is another more distant relative of this family of proteins encoded by *YEL020c* which is 23% identical to *Pdc1p*. At present its function is completely unknown.

The observation that substantial quantities of α -methylbutyrate were produced non-enzymically from α -methylbutyraldehyde may have significance in commercial yeast fermentations especially the brewing of beer. Active amyl alcohol and esters derived from it are important flavor compounds, whereas α -methylbutyrate has a most unpleasant odor. Thus, attempts to increase the concentration of the desirable active

amyl alcohol could have the entirely opposite effect by causing the concomitant increase in the undesirable α -methylbutyrate.

The present study and our previous studies (1, 2) on the catabolism of the three branched-chain amino acids have found no role for branched-chain α -ketoacid dehydrogenase when yeast is cultured in minimal media. At first sight this seems to make the physiological role of this enzyme more of a mystery. In fact, the contrary is true because it offers an explanation for one of Ehrlich's long standing conundrums. Ehrlich could not explain why in complex media there was no correlation between the kinetics of amino acid utilization and fusel alcohol formation (3, 4). It is now known that the highest specific activity of branched-chain α -ketoacid dehydrogenase is observed in cells growing in complex media when glycerol is the carbon source (9). In minimal media where a branched-chain amino acid is the sole nitrogen source, branched-chain α -ketoacid dehydrogenase is not involved in fusel alcohol formation. Hence, in complex media catabolism of the branched-chain amino acids proceeds via branched-chain α -ketoacid dehydrogenase, and fusel alcohols are not formed.

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