The Catabolism of Amino Acids to Long Chain and Complex Alcohols in *Saccharomyces cerevisiae* *

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The catabolism of phenylalanine to 2-phenylethanol and of tryptophan to tryptophol were studied by 13C NMR spectroscopy and gas chromatography-mass spectrometry. Phenylalanine and tryptophan are first deaminated (to 3-phenylpyruvate and 3-indolepyruvate, respectively) and then decarboxylated. This decarboxylation can be effected by any of Pdc1p, Pdc5p, Pdc6p, or Ydr380wp; Ydl080cp has no role in the catabolism of either amino acid. We also report that in leucine catabolism Ydr380wp is the minor decarboxylase. Hence, all amino acid catabolic pathways studied to date use a subtly different spectrum of decarboxylases from the five-membered family that comprises Pdc1p, Pdc5p, Pdc6p, Ydl080cp, and Ydr380wp. Using strains carrying all possible combinations of mutations affecting the seven AAD genes (putative aryl alcohol dehydrogenases), five ADH genes, and SFA1, showed that the final step of amino acid catabolism (conversion of an aldehyde to a long chain or complex alcohol) can be accomplished by any one of the ethanol dehydrogenases (Adh1p, Adh2p, Adh3p, Adh4p, Adh5p) or by Sfa1p (formaldehyde dehydrogenase.)

It is well known that *Saccharomyces cerevisiae* converts sugars to ethanol using the glycolytic pathway. In addition, it also produces a number of long chain and complex alcohols including isovalryl alcohol, isobutanol, active amyl alcohol, 2-phenylethanol, and tryptophol (1). These are all important flavor and aroma compounds in yeast-fermented products and have interesting organoleptic properties in their own right, many of which are both concentration- and context-dependent. These long chain and complex alcohols are the end products of amino acid catabolism. In this respect the pathways used by *S. cerevisiae* are very different from those in other eukaryotes that have been well understood for many years (2).

We have sought to discover the genes and enzymes used by *S. cerevisiae* in the catabolism of leucine to isovalryl alcohol (3), valine to isobutanol (4), and isoleucine to active amyl alcohol (5). In all cases, the general sequence of biochemical reactions is similar, but the details for the formation of the individual alcohols are surprisingly different. The branched-chain amino acids are first deaminated to the corresponding α-ketoacids (α-ketoisocaproic acid from leucine, α-ketoisovaleric acid from valine, and α-keto-β-methylvaleric acid from isoleucine). There are significant differences in the way each α-ketoacid is subsequently decarboxylated. In the leucine degradation pathway the major decarboxylase is encoded by YDL080c. Consequently, we refer to this open reading frame as KID1 for ketoisocaprate decarboxylase 1 (3). In valine degradation any one of the three isoforms of pyruvate decarboxylase encoded by PDC1, PDC5, and PDC6 will decarboxylate α-ketoisovalerate (4), and in isoleucine catabolism any one of the family of decarboxylases encoded by PDC1, PDC5, PDC6, YDL080c, or YDR380w is sufficient for the conversion of isoleucine to active amyl alcohol (5).

There are three important questions that remain unanswered. First, our earlier work had shown that the YDL080c-encoded decarboxylase was the major route of decarboxylation of α-ketoisocaproate but that there was at least one other unidentified decarboxylase involved in leucine catabolism. This enzyme should be identified, and its metabolic contribution should be quantified. Second, other than the fact that tryptophan is the precursor of tryptophol, and phenylalanine is the precursor of 2-phenylethanol (1), little else is known about the genes and enzyme-catalyzed steps involved in the catabolism of these two aromatic amino acids in *S. cerevisiae* except that Ydr380wp has been suggested as a potential indolepyruvate decarboxylase (the enzyme that would be required for the conversion of indolepyruvate to indole acetaldehyde if tryptophan is catabolized in an analogous way to the branched-chain amino acids) (6). Third, the final stage of long chain and complex alcohol formation has always been assumed to be an alcohol dehydrogenase-catalyzed step (1), but this has never been rigorously examined. Because yeast has 20 genes that could potentially encode this activity, seven putative aryl alcohol dehydrogenase genes (7, 8) and 13 other alcohol dehydrogenase genes, ADH1, ADH2, ADH3, ADH4, ADH5, SFA1, BDH1, putative BDH2 (YAL061w), SOR1, putative SOR2 (YDL246c), XDH1, the cinnamyl alcohol dehydrogenase *CDH1* (YCR105w), and the recently proposed *ADH6* (YMR318c) (9), it is also necessary to discover which of these genes products are required for long chain and complex alcohol formation. This paper addresses all three questions.

**EXPERIMENTAL PROCEDURES**

*Strains, Media, and Cultural Conditions—* The strains used are shown in Table I. IWD72 is the wild-type strain that we have used for all studies of amino acid catabolism. Strain YPH499 is the wild-type parent of strain AAD7. Standard genetic techniques were used in all cases (11, 12). Strain 59.2.1 was produced by crossing strain Y04623 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 adh4::kanMX4 from EUROSCARF) with strain 58.3.4 (MATa ura3Δ2 his3Δ1 leu2Δ0 met15Δ0 his3Δ200 leu2Δ1). Strain 58.3.4 had been produced in an earlier cross between IWD72 and YPH499. Strain 60.3.3 was produced by mating strain 900–17–101a to strain 59.2.1 and sporulation of the resultant diploid. Strain 61.1.1 was produced by sporulating strain Y26236 (MATaXMATa his3Δ1 his3Δ1 leu2Δ0/1 leu2Δ0 lys2Δ0/LYS2 MET15/ met15Δ0 ura3Δ0/ura3Δ0 adh1::kanMX4/ADH1 from EUROSCARF) with strain 61.1.1 (MATaMATa his3Δ1 his3Δ1/1 leu2Δ0/1 leu2Δ0 lys2Δ0/LYS2 MET15/ met15Δ0 ura3Δ0/ura3Δ0 adh1::kanMX4/ADH1 from EUROSCARF).

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and then dissection of the ascospores. Strain ESH380w was produced from 0 to 1000 Hz because of the adjacent labeled C-2). There

Yielded products that were unique to each disruption allele.

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Yeast strains used

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| 51.1.3 | MATa ura3 ydl080c::kanMX4 | (5) |
| 55.2.1 | MATa ade8 leu2Δ ydr380w::kanMX4 | (5) |
| 57.2.3 | MATa ade8 leu2 ura3 ydr380w::kanMX4 ydr380w::kanMX4 | (5) |
| YSH5.127.17C | MATa leu2-3,112 ura3-52, trp1-86 pdcδ::LEU2 pdcδ::URA3 pdcδ6::TRP1 GAL | S. Hohmann

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

The YDR380w-encoded Decarboxylase Makes a Minor Contribution in Leucine Catabolism—Strain 56.9.2 (pdcδ pdcδ ydl080c ydr380w) was precultured in yeast extract-potato-ethanol medium for 48 h when its A600 nm had reached 8.0. An aliquot (1 ml) of this culture was inoculated into ethanol minimal medium in which leucine (2%) (w/v) was the sole source of nitrogen, and the cells were then cultured for 407 h by which time the A600 nm was 12.2, corresponding to stationary phase for this strain in this medium. The concentration of isomyl alcohol remained zero throughout the experiment. When strain JRD815–1.2 (pdcδ ydl080c ydr380w) was cultured in the same medium for a similar time the concentration of isomyl alcohol fell to ~6% of that produced by its parent YSH5.127.17C (pdcδ pdcδ ydl080c) (3). Thus, the ydl080c mutation had caused a 94% reduction in isomyl alcohol levels (3). This indicates that the YDL080c gene product is responsible for 94% of the decarboxylation of α-ketosidroacipic acid and that YDR380wp is the minor decarboxylase being responsible for only 6% of the flux in leucine degradation.

Phenylalanine Catabolism in a Wild-type Strain—Fig. 1 shows the 13C NMR spectrum of a culture supernatant of wild-type strain IWD72 that had been cultured for 24 h in a minimal medium in which glucose was the carbon source and [2-13C]phenylalanine was the sole nitrogen source. The largest signal was C-2 of phenylalanine (the 13C-labeled substrate). A number of resonances were observed because of natural abundance in the phenylalanine substrate: C-1 (at 174 ppm appearing as a doublet J = 54 Hz because of the adjacent labeled C-2), C-1 of the phenyl ring (135.1 ppm), C-2, C-3, C-4, C-5, and C-6 of the phenyl ring (129.3 ppm, 129.1 ppm, 128.6 ppm, 127.6 ppm, 126.6 ppm, and 126.4 ppm), and C-3 (at 36.4 ppm appearing as a doublet J = 34 Hz because of the adjacent labeled C-2). There were no natural abundance signals because of glucose indicat-

RESULTS

Fig. 1 shows the 13C NMR spectrum of a culture of strain IWD72, which was grown in minimal medium containing glucose as the carbon source and [1-13C]phenylalanine as the sole nitrogen source. The largest signal was C-2 of phenylalanine (the 13C-labeled substrate). A number of resonances were observed because of natural abundance in the phenylalanine substrate: C-1 (at 174 ppm appearing as a doublet J = 54 Hz because of the adjacent labeled C-2), C-1 of the phenyl ring (135.1 ppm), C-2, C-3, C-4, C-5, and C-6 of the phenyl ring (129.3 ppm, 129.1 ppm, 128.6 ppm, 127.6 ppm, 126.6 ppm, and 126.4 ppm), and C-3 (at 36.4 ppm appearing as a doublet J = 34 Hz because of the adjacent labeled C-2). There were no natural abundance signals because of glucose indicat-
ing that it had all been consumed, but the resonances at 57.5 ppm and 16.8 ppm were because of C-1 and C-2 (respectively) of ethanol, which had been produced from the glucose. Another resonance that was observed because of natural abundance was C-1 of the phenyl ring of 2-phenylethanol at 139.1 ppm, which, along with the large resonance at 62.6 ppm, because of C-1 of 2-phenylethanol, and the smaller doublet at 37.8 ppm (J = 37 Hz), the C-2 of 2-phenylethanol, indicates that large quantities of this compound were produced from the [2-13C]phenylalanine substrate. Other important resonances identified were C-2 of 3-phenylpyruvate (204.2 ppm) and C-2 of 3-phenyllactate (73.4 ppm). A very small resonance at 214.7 ppm and a larger one at 64.1 ppm remain unidentified. The 3-phenyllactate (73.4 ppm). A very small resonance at 214.7 ppm and a larger one at 64.1 ppm, because of C-2 of 3-phenylpyruvate; A-1, C-2 of the phenyl ring of phenylalanine; V, C-3, C-3, C-4, C-5, and C-6 of the phenyl ring of phenylalanine; PL2, C-2 of 3-phenyllactate; PE1, PE2, C-1, and C-2 (respectively) of 2-phenylethanol; E1, E2, C-1, and C-2 (respectively) of ethanol.

Because the various strains grew to different extents, comparisons of the amount of 2-phenylethanol produced are better on a per cell basis rather than on the basis of absolute concentration in the growth medium. Table II shows that a significant reduction in 2-phenylethanol occurred in the pdc1 pdc5 pdc6 ydr380w strain (ESH380w). The pdc1 pdc5 pdc6 triple mutant (YSH5.127.17C) and the ydr380w single mutant (55.2.1) did not show similar reductions in the amount of 2-phenylethanol synthesized. This indicates that the decarboxylation of 3-phenylpyruvate can proceed if any one of the four genes, PDC1, PDC5, PDC6, or YDR380w, is functional. A mutation in lpd1 mutant 53.1.4 that is defective in lipoamide dehydrogenase produced wild-type levels of 2-phenylethanol. Lipoamide dehydrogenase is an essential component of branched-chain α-ketoacid dehydrogenase, pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and glycine decarboxylase (13–16). Hence, because strain 53.1.4 lacks branched-chain α-ketoacid dehydrogenase, it can be concluded that this activity is not involved in the formation of 2-phenylethanol from phenylalanine.

**Tryptophan Catabolism in a Wild-type Strain**—Fig. 4 shows the 13C NMR spectrum of a culture supernatant of wild-type strain IWD72 that had been cultured for 24 h in a minimal medium in which glucose was the carbon source and [U-13C]phenylalanine was the sole nitrogen source. All of the strains were capable of using phenylalanine as the sole source of nitrogen (Fig. 3), and all had reached stationary phase by 100 h. Strain 53.1.4 (lpd1) grew conspicuously less well than all of the other strains. As noted previously, strains carrying lpd1 mutations have reduced energy production and, consequently, reduced growth on glucose. The various minimal media compared with the wild-type (4, 15).
U-$^{15}$N-tryptophan was the sole nitrogen source. The two $^{15}$N atoms showed negligible coupling to $^{13}$C atoms, and the NMR spectrum was effectively the same as if the tryptophan was labeled only with $^{13}$C atoms. All of the C atoms of tryptophan were observed: C-1 (at 174.5 ppm appearing as a doublet J = 54 Hz), C-7' of the indole ring (a triplet at 136 ppm), the C-6', C-5', C-4', C-3a', C-2a', C-3', and C-2' atoms in the indole ring (multiplets at 126.6, 125.2, 122.0, 119.4, 118.4, 112.0, and 107.5 ppm), C-2 (a double doublet at 55.2 ppm), and C-3 (a double doublet at 26.4 ppm). As in the phenylalanine experiment, there were no natural abundance signals because of glucose indicating that it had all been consumed, but the resonances at 57.5 and 16.8 ppm (C-1 and C-2, respectively, of ethanol, produced from the glucose) were evident. Also evident were the C-1 (a doublet J = 37 Hz at 61.8 ppm) and C-2 (a double doublet at 27.3 ppm) of tryptophol, which had been produced from the tryptophan. The small resonance at 22.5 ppm could not be identified. The most likely candidate was thought to be the ethyl ester of tryptophan. $S$. cerevisiae forms a wide variety of esters, the quantities formed being largely determined by the concentrations of acids and alcohols present (17). With the high concentrations of tryptophan and ethanol observed in this experiment, the formation of tryptophan ethyl ester would not have been a surprise. However, examination of the spectrum of the $b$ona $f$ide compound showed that it was not tryptophan ethyl ester. The resonance at 62.6 ppm is attributed to C-1 of 2-phenylethanol and is a singlet indicating that it was observed because of natural abundance and thus was not derived directly from tryptophan. The significance of this is further considered below.

From the metabolites identified it is clear that tryptophan catabolism to tryptophol must proceed as shown in Fig. 5. The fact that no signals were observed because of 3-indolepyruvate indicates that the pool size of this compound must be extremely low, and/or its subsequent decarboxylation is very rapid. This is consistent with the fact that no 3-indolelactate was observed although the absence of this compound may be because there is no enzyme in yeast that is capable of reducing 3-indolepyruvate to 3-indolelactate.

Ydl080cp also Has No Role in Tryptophan Catabolism—The same set of mutants used to study phenylalanine catabolism were also analyzed for their ability to grow and produce tryptophol in minimal medium in which tryptophan was the sole nitrogen source. All of the strains were capable of using tryptophan as the sole source of nitrogen (Fig. 6), and all except ESH380w had reached stationary phase by 144 h. Strain ESH380w (pdc1 pdc5 pdc6 ydr380w) had a very prolonged deceleration phase and did not reach stationary phase until 550 h. Upon inspecting the final cell density, it can be seen that there were two groups of strains. The majority, including the wild-type, reached $A_{600 \text{ nm}}$ in the range from 6 to 7, whereas four strains (ESH380w, 55.2.1, 57.2.13, and 53.1.4) only reached half that density. As before, to allow for the fact that the various strains grew to different extents, comparisons of the amount of tryptophol produced are better made on a per cell basis rather than simply on the concentration in the growth medium. Table III shows that most of the strains produced roughly similar amounts of tryptophol to the wild-type, but strains ESH380w (pdc1 pdc5 pdc6 ydr380w) and 56.9.2 (pdc1 pdc5 pdc6 ydl080c ydr380w) did not produce this compound. Because the pdc1 pdc5 pdc6 mutant YSH5.127.17C and the ydr380w single mutant 55.2.1 did not produce lowered levels of tryptophol, and ydl080c mutations ( singly or in combination with other mutations) did not result in a lowering of tryptophol levels, it is apparent that tryptophan can be catabolized to tryptophol if any one of the four genes, PDC1, PDC5, PDC6, or YDR380W, is functional and that, as with phenylalanine catabolism in this yeast, YDL080c has no role.

Tryptophol levels were also reduced in the lpd1 mutant 53.1.4. This could be interpreted to mean that lipoamide dehydrogenase is required for tryptophan catabolism to tryptophol and that there is an alternative metabolic route involving one or more of branched-chain $\alpha$-ketoacid dehydrogenase, pyruvate dehydrogenase, $\alpha$-ketoisocaproate dehydrogenase, or glycine decarboxylase. However, we do not believe this to be the case for three reasons. First, the NMR and GC-MS analyses of the wild-type strain provide no evidence of alternative intermediates or end products. Second, the lpd1 mutant does not accumulate any intermediates that could be considered to be derived from tryptophan. Third, the tryptophol level produced by strain 53.1.4 is not reduced as drastically as in strains ESH380w and 56.9.2. On a per cell basis it is still 31% of the level produced by the wild-type in the same (glucose minimal)
We suspect that the reduced tryptophol formation in strain 53.1.4 is an indirect consequence of both its impaired energy metabolism and the fact that the absence of pyruvate dehydrogenase and \(a\)-ketoglutarate dehydrogenase activities will result in shortages of \(\beta\)-ketoglutarate, which is required in the initial deamination of tryptophan. The \(lpd1\) mutant uniquely did not consume all of the tryptophan by the end of the experiment.

The NMR analysis of the catabolism of \(^{13}\text{C}\)-labeled tryptophan indicated the presence of unlabeled 2-phenylethanol. The results in Table III that were obtained using GC-MS confirm the presence of 2-phenylethanol at concentrations that were between one-third and one-half of the concentrations of tryptophol. The levels of 2-phenylethanol produced by cells grown on tryptophan as the sole nitrogen source were about one-tenth of the levels produced when the cells were grown on phenylalanine as the sole nitrogen source. This indicates wasteful catabolism of phenylalanine when yeast is growing on tryptophan as the sole nitrogen source.

**TABLE II**

| Strain         | Carbon source | Relevant genotype | 2-Phenylethanol  | 2-Phenylethanol |
|----------------|---------------|-------------------|------------------|----------------|
|                |               |                   | \(\mu\text{g/ml}\) | \(\mu\text{g/10}^6\text{ cells}\) |
| 53.1.3         | Ethanol       | ydl080c           | 1942             | 15.3           |
| 55.2.1         | Ethanol       | ydr380w           | 1796             | 17.9           |
| 57.2.13        | Ethanol       | ydl080c ydr380w   | 1856             | 19.6           |
| YSH5.127.17C   | Ethanol       | pdc1 pdc5 pdc6    | 1609             | 14.5           |
| JRD615–1.2     | Ethanol       | pdc1 pdc5 pdc6 ydl080c | 1460              | 11.7           |
| ESH380w        | Ethanol       | pdc1 pdc5 pdc6 ydr380w | 10                  | 0.2            |
| 56.9.2         | Ethanol       | pdc1 pdc5 pdc6 ydl080c ydr380w | 9                  | 0.1            |
| 53.1.4         | Glucose       | lpd1              | 380              | 17.3           |
| IWD72          | Ethanol       | Wild-type         | 3315             | 21.3           |
| IWD72          | Glucose       | Wild-type         | 3416             | 21.9           |

**FIG. 4**. The \(^{13}\text{C}\) NMR spectrum of the wild-type strain in \([U-^{13}\text{C}]_1, U-^{15}\text{N}_2\)tryptophan. The wild-type strain IWD72 was cultured for 24 h in glucose minimal medium containing \([U-^{13}\text{C}]_1, U-^{15}\text{N}_2\)tryptophan as the sole nitrogen source. The resonances marked 1, 2, and 3 are C-1 to C-3 (respectively) of tryptophan. The other resonances identified are 7, 7' of the indole ring of tryptophan; \(IR\), all of the other C atoms in the indole ring of tryptophan; \(PE1\), C-1 of 2-phenylethanol; \(T1\), \(T2\), C-1, and C-2 (respectively) of tryptophol; \(E1\), \(E2\), C-1, and C-2 (respectively) of ethanol.

**FIG. 5**. The pathway of tryptophan catabolism in \(S.\ cervisiae\).
all of which are of unknown function (7, 8), and 13 other alcohol dehydrogenases. Seven of these can be eliminated after a consideration of their properties. For example, BDH1 (YAL060w) has been shown recently (18) to be involved in the formation of 2,3-butanediol. On the basis of similarity it is safe to assume that YAL061W can be called BDH2 and that it has a similar activity. SOR1 (YJR159W), SOR2 (YDL246C), and XDH1 (YLR070C) encode sugar alcohol dehydrogenases (19, 20), which are not the type of enzyme activities sought in this investigation. CDH1 (YCR105W) and ADH6 (YMR318C) encode NADPH-dependent enzymes and ADH6 is the best known. Adh1p is the main cytosolic enzyme involved in the formation of ethanol during glycolysis. Adh2p, which is also cytosolic, is the glucose-repressed enzyme that is needed for growth on ethanol. Adh3p is mitochondrial, it is induced on glucose, and its role is not really understood. Recent work from Pronk and co-workers (21) suggest it is involved in a redox shuttle. Adh4p is present at only very low levels in most laboratory strains, but is plentiful in brewing strains (22). Adh5p was discovered by genome sequencing, and its role is unexplained. Sfa1p is part of a bifunctional enzyme that has both glutathione-dependent formaldehyde dehydrogenase activity (the gene name comes from sensitive to formaldelyde, which is the phenotype of mutants affected in this gene); it is also described as being capable of catalyzing the breakdown of long chain alcohols (23). Hence, each of Adh1p-Adh5p and Sfa1p has at least one feature suggesting that it could be responsible for the synthesis of long chain alcohols. For example, Adh1p, Adh2p, and Adh3p all require zinc, and brewers have known for years that the concentration of zinc in the wort affects the concentrations of long chain alcohols that are formed. Alternatively, Adh3p and Adh4p appear to be good candidates as no certain role has yet been ascribed to them. Finally, because it has been shown that Sfa1p can degrade long chain alcohols in vitro, perhaps its true role in vivo is the formation of these compounds.

FIG. 6. The growth of various strains in minimal medium with tryptophan as the sole nitrogen source. The carbon source was 2% ethanol except where indicated. \(\Delta\) with solid line, IWD72 (wild-type); \(\bullet\) with solid line, IWD72 glucose as carbon source; \(\triangle\) with solid line, ESH380w; \(\square\) with solid line, YSH5.127.-17C; \(\diamondsuit\) with dashed line, YSH5.127.17C; \(\triangleleft\) with dashed line, 55.1.3; \(\ast\) with solid line, JRD815—1.2; \(\mathbf{I}\) with dotted line, 53.1.4. Glucose as carbon source.

| Table III |
|---|
| The amount of tryptophol and 2-phenylethanol produced by various yeast strains growing in a minimal medium with tryptophan as sole source of nitrogen |

All pdc1 pdc5 pdc6 triple mutants cannot utilize glucose; these had ethanol (2%) as carbon source. A lpd1 mutant cannot use ethanol; this strain had glucose (2%) as carbon source. Tryptophol and 2-phenylethanol were determined by GC-MS when each strain had reached stationary phase. The values are the means of two separate experiments. Differences between samples were <4%.

| Strain      | Carbon source | Relevant genotype | Tryptophol | 2-Phenylethanol | 2-Phenylethanol |
|-------------|---------------|-------------------|------------|----------------|----------------|
|             |               |                   | \(\mu\)g/ml | \(10^6\) cells | \(10^6\) cells |
| 51.1.3      | Ethanol       | ydl080c           | 164        | 27            | 105            | 1.7            |
| 55.2.1      | Ethanol       | ydr380w           | 110        | 3.0           | 57             | 1.6            |
| 57.2.13     | Ethanol       | ydl080c ydr380w   | 149        | 3.8           | 107            | 2.5            |
| YSH5.127.17C| Ethanol       | pdc1 pdc5 pdc6    | 149        | 3.8           | 107            | 2.5            |
| JRD815—1.2 | Ethanol       | pdc1 pdc5 pdc6 ydl080c | 376 | 4.9 | 205 | 2.7 |
| ESH380w     | Ethanol       | pdc1 pdc5 pdc6 ydr380w | 0 | 0.0 | 2 | 0.1 |
| 56.9.2      | Ethanol       | pdc1 pdc5 pdc6 ydl080c | 0 | 0.0 | 2 | 0.1 |
| 53.1.4      | Glucose       | lpd1              | 59         | 1.6           | 44             | 1.2            |
| IWD72       | Ethanol       | Wild-type         | 478        | 7.1           | 159            | 2.4            |
| IWD72       | Glucose       | Wild-type         | 309        | 5.1           | 99             | 1.7            |

| Table IV |
|---|
| The amount of isoamyl alcohol produced by various yeast strains growing in glucose minimal medium with leucine as sole source of nitrogen |

Isoamyl alcohol was determined by GC-MS when each strain had reached stationary phase. The values are the means of two separate experiments. Differences between samples were <5%.

| Strain      | Relevant genotype | Isoamyl alcohol | Isoamyl alcohol |
|-------------|-------------------|-----------------|-----------------|
|             |                   | \(\mu\)g/ml     | \(10^6\) cells |
| 61.1.1      | adh1              | 162             | 4.9             |
| Y00891      | adh2              | 384             | 6.0             |
| Y06217      | adh3              | 308             | 6.1             |
| 59.2.1      | adh4              | 246             | 9.4             |
| Y03284      | adh5              | 451             | 6.8             |
| 301.10a     | adh1 adh2        | 87              | 6.7             |
| 900-17-101a | adh1 adh2 adh3   | 101             | 2.3             |
| 60.3.5      | adh1 adh2 adh3 adh4 | 509 | 6.1 |
| 62.3.17     | adh1 adh2 adh3 adh4 adh5 | 491 | 9.0 |
| Y03866      | sfa1             | 534             | 7.7             |
| 66.1.3      | adh1 adh2 adh3 adh4 sfa1 | 479 | 7.0 |
| AAD7x       | adad3 adad4 adad6 adad10 adad14 adad15 adad16 | 308 | 5.8 |
| IWD72       | Wild-type        | 716             | 5.4             |
To determine which alcohol dehydrogenase is/are required for the formation of fusel alcohols we used a strain mutated in all seven of its AAD genes and constructed other strains containing all possible combinations of single and multiple mutations affecting the six alcohol dehydrogenases. These strains were then grown in glucose minimal medium containing a single amino acid as the sole nitrogen source and analyzed for the ability to form the alcohol end product corresponding to the amino acid supplied. Table IV shows the amount of isoamyl alcohol produced when a selection of these strains were grown on leucine as the sole nitrogen source. All of the strains produced isoamyl alcohol. Equivalent results were obtained using other combinations of mutations and different single amino acid nitrogen sources. It appears that the AAD genes have no role in long chain or complex alcohol formation. Using both conventional crosses and by trying to transform strains carrying five alcohol dehydrogenase mutations, it proved impossible to construct a haploid that had mutations in all six alcohol dehydrogenase genes. From this we conclude that as long as yeast has one enzyme out of Adh1p-Adh5p or Sfa1p that is functional, it is viable and able to form long chain and complex alcohols.

**DISCUSSION**

Our previous study of leucine catabolism had identified Ydl080cp as the major decarboxylase, because mutation of this open reading frame alone led to an almost total (94%) abolition of the formation of isoamyl alcohol (3). The current study has identified Ydr380wp as the minor decarboxylase occurring for only 6% of the flux. When both YDL080c and YDR380w are mutated a haploid strain of *S. cerevisiae* growing in minimal medium with leucine as the sole nitrogen source makes no isoamyl alcohol indicating that no other pathway exists for the synthesis of this compound.

This study has shown that in *S. cerevisiae* the catabolism of phenylalanine to 2-phenylethanol and of tryptophan to 2-phenylethanol are both accomplished in a similar way corresponding to what has been called the Ehrlich pathway (1). Based on the results obtained from strains in different yeast species, it is often asserted (24) that yeasts catabolize phenylalanine via *trans*-cinnamate to benzochloroacetate and that the benzene ring is subsequently opened to give maleylacetate, which is then suppressed reduced to 3-ketoisocaprate. The same authors state that tryptophan degradation involves ring opening by a dioxygenase to yield *N*-formyl-*k*ynurenine. Deformylation is said to yield *k*ynurenine, which then has an alanine moiety removed to produce anthranilate. The pathway subsequently involves *cis*-dihydroxybenzoate, catechol, *cis*-isocitrate, and finally 3-ketoisocaprate. Evidence for both pathways comes mainly from *Trichosporon cutaneum* (25, 26). Our NMR study showed no evidence of any of the intermediates of either of these pathways. Furthermore, 3-ketoisocaprate, the supposed end product of both phenylalanine and tryptophan catabolism, would give a clear signal in the region 200–210 ppm because of the C=O group. There was no such resonance in either spectrum. The only conclusion possible is that in *S. cerevisiae* phenylalanine and tryptophan are catabolized according to the Ehrlich pathway.

In both phenylalanine and tryptophan catabolism the decarboxylation step is carried out by any one of the gene products of *PDC1*, *PDC5*, *PDC6*, or YDR380w. YDL080c has no part in the catabolism of either of these amino acids. Thus, it is apparent that in each of the amino acid catabolic pathways that we have studied, the crucial decarboxylation step is accomplished in subtly different ways. In leucine catabolism YDL080c is the major decarboxylase, and YDR380wp is the minor decarboxylase. In valine catabolism any one of the pyruvate decarboxylase isozymes (Pdc1p, Pdc5p, or Pdc6p) can perform the decarboxylation of α-ketoisovaleric acid (4), whereas in isoleucine catabolism any one of the five-membered family of decarboxylases (Pdc1p, Pdc5p, Pdc6p, Ydl080cp, or Ydr380wp) is sufficient. In complete contrast, for catabolism of the aromatic amino acids phenylalanine and tryptophan YDL080c is irrelevant; it is not needed for these two pathways.

This study showed that any one of six alcohol dehydrogenases (encoded by *ADH1*, *ADH2*, *ADH3*, *ADH4*, *ADH5*, or *SFA1*) is sufficient for the final stage of long chain or complex alcohol formation in *S. cerevisiae*. Of course, this does not mean that every 5-fold alcohol dehydrogenase mutant will proliferate and produce fusel alcohols under all conditions. For example, an *adhl adh3 adh4 adh5 sfa1* mutant will not grow in high concentrations of glucose, because the remaining alcohol dehydrogenase (*Adh2p*) is repressed by glucose. More than 40 years ago, well before the genome and proteome of this yeast were fully characterized, it had been concluded that the final step in fusel alcohol formation was catalyzed by either of the enzymes that are now known as Adh1p or Adh2p (27). This correct but incomplete conclusion was made without a knowledge of all of the alcohol dehydrogenases that yeast has. The functions of the seven AAD genes remain unknown.

In *S. cerevisiae* catabolism of the three branched-chain amino acids (leucine, valine, and isoleucine) and the two aromatic amino acids (phenylalanine and tryptophan) proceeds essentially as Ehrlich proposed nearly 100 years ago (28). However, it is much more complex than Ehrlich ever imagined, because yeast uses at least three aminotransferases, five decarboxylases, and six alcohol dehydrogenases. The precise combination of enzymes used at a particular time depends upon the amino acid, the carbon source, and the stage of growth of the culture. In our experience, the major specificity resides with the decarboxylases. It is the regulation of these decarboxylases that is the current focus of our interest.

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Saccharomyces cerevisiae
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J. Biol. Chem. 2003, 278:8028-8034.
doi: 10.1074/jbc.M211914200 originally published online December 23, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M211914200

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