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Regulation of Leucine Catabolism in *Pseudomonas putida*

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The generation time of *Pseudomonas putida* with L-leucine was 20 h in synthetic media but only 3 h with D-leucine. Slow growth in the presence of L-leucine was partially overcome by addition of 0.1 mM amounts of either D-valine, L-valine, or 2-ketoisovalerate. The activities of five enzymes which take part in the oxidation of leucine by *P. putida* were measured under various conditions of growth. Four enzymes were induced by growth with DL-leucine as sole source of carbon: D-amino acid dehydrogenase, branched-chain keto acid dehydrogenase, 3-methylcrotonyl-coenzyme A carboxylase, and 3-hydroxy-3-methylglutaryl-coenzyme A lyase. The segment of the pathway required for oxidation of 3-methylcrotonate was induced by growth on isovalerate or 3-methylcrotonate without formation of the preceding enzymes. The synthesis of carboxylase and lyase appeared to have been repressed by the addition of L-glutamate or glucose to cells growing on DL-leucine as the sole carbon source. Mutants unable to grow at the expense of isovalerate had reduced levels of carboxylase and lyase, whereas the levels of three enzymes common to the catabolism of all three branched-chain amino acids and those of two isoleucine catabolic enzymes were normal.

The proposed pathway for the oxidation of leucine by *Pseudomonas putida* is shown in Fig. 1. Three enzymes, D-amino acid dehydrogenase, branched-chain amino acid transaminase, and branched-chain keto acid dehydrogenase, have been identified previously as necessary for the oxidation of D- and L-leucine in *P. putida*. Partially purified D-amino acid dehydrogenase from *P. aeruginosa* was able to deaminate the D-isomers of all three branched-chain amino acids (13). Purified branched-chain amino acid transaminase from *P. aeruginosa* deaminated L-isomers of all three branched-chain amino acids (17), and a mutation which affected the transaminase was associated with the concomitant loss of ability to grow at the expense of all three branched-chain amino acids (15). Another class of mutants showed a complete loss of branched-chain keto acid dehydrogenase and loss of ability to grow on the branched-chain amino acids as well as their corresponding keto acids as carbon sources (15).

A carbon dioxide-fixing enzyme, 3-methylcrotonyl-coenzyme A (CoA) carboxylase, was first reported by Lynen et al. (12) to be necessary for the oxidation of leucine and isovalerate in species of *Mycobacterium* and *Achromobacter* isolated from soil. Rilling and Coon (19) have demonstrated the carboxylation of 3-methylcrotonyl-CoA by extracts of *Pseudomonas oleovorans*, classified by Stanier as a member of *P. putida* biotype (25). The final enzyme unique to leucine catabolism, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) lyase, has been reported in bacteria only in extracts of an actinomycete grown on mevalonic acid (21). These extracts were capable of oxidizing mevalonic acid to HMG-CoA.

The purpose of this paper is to report studies which confirm the pathway of leucine catabolism in *P. putida* and to present studies on the regulation of leucine catabolism.

MATERIALS AND METHODS

Organisms. *P. putida* strain PpG2 (ATCC 23287), strain PpG701, a streptomycin-resistant, camphor-negative segregant derived from PpG1, and strain PpG736, an isobutyrate-negative derivative of PpG701, were obtained from H. Dunn and I. C. Gunsalus at the University of Illinois.

Mutants of PpG736 were obtained by treatment with nitrosoguanidine, as described by Martin et al. (15), and by selection for mutants unable to grow with isovalerate 0.3% using the penicillin and D-cycloserine enrichment procedure of Ornston et al. (18).

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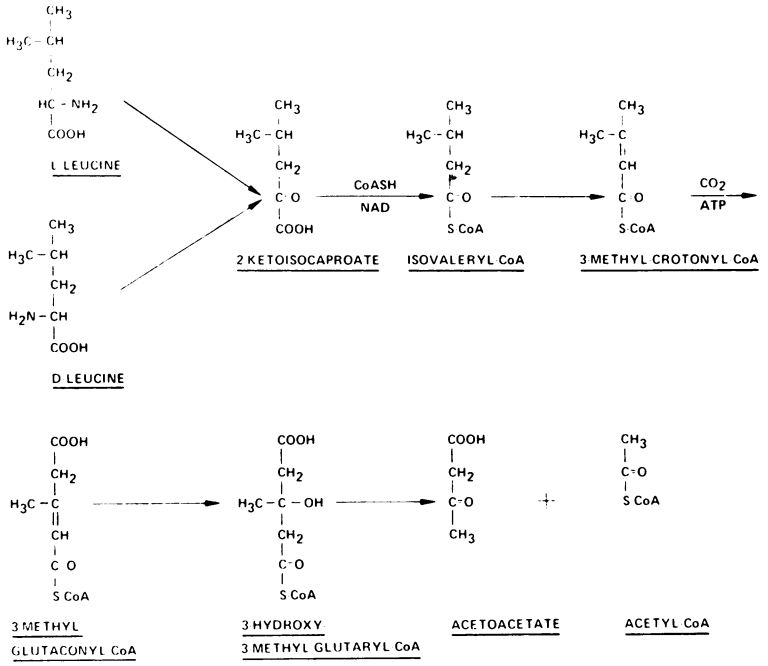

FIG. 1. *Pathway for the metabolism of* D- *and* L-*leucine in pseudomonads.*

A mutant phenotype is indicated by an isolation number. The nomenclature used follows the recommendation of Demerec et al. (3). Phenotype abbreviations, rather than genotype symbols, have been used to identify mutants because the genes affected by the mutations have not been determined. For the sake of clarity and completeness, phenotype abbreviations have also been assigned (Fig. 2) for the traits expected of mutants which have, as yet, not been isolated.

Stock cultures were maintained on tryptose-phosphate agar slants and were transferred once a month.

Growth conditions. *P. putida* was grown at 30 C in the basal medium of Jacobson et al. (9), with carbon sources added at indicated concentrations. The pH was adjusted to 7.0 as necessary. Cultures were shaken on a New Brunswick gyratory water bath-shaker (model G76).

For the determination of generation times, 1.0 ml of a culture grown overnight on 0.3% L-glutamate was inoculated into 50 ml of medium in a 200-ml side-arm flask. Optical densities were read at 660 nm with a Bausch and Lomb Spectronic 20 spectrophotometer.

For the catabolite repression experiment, 50 ml of basal medium containing 15 mM DL-leucine was inoculated from slants and grown overnight. Portions of the 50-ml culture were transferred to 500-ml flasks of the same medium, which were shaken until log growth. At this time, sterile glucose in 5 ml of water was added to bring the concentration of glucose to 22 mM. Optical densities were read at 660 nm on a Coleman 124 double-beam spectrophotometer, with uninoculated medium as the reference. Culture samples were centrifuged at 4 C and the cell pellet was quickly frozen. On the following day, cell-free extracts were prepared for enzyme assay.

Extraction of enzymes for assay. Enzyme extracts were prepared by suspending cells in freshly prepared 0.05 M potassium phosphate buffer (pH 7.5) containing 2 mM dithiothreitol. The suspension was subjected to sonic disruption, in 3- to 6-ml portions, with a Branson model S75 Sonifier for 60 s, and centrifuged at 10,000 $\times$ *g* for 15 min at 4 C. The supernatant was used as the source of enzyme. Cell-free extracts were stored in ice, and enzymes were assayed within 8 h of preparation. Protein concentrations were determined by the method of Warburg and Christian (28).

Synthesis of substrates. 3-Hydroxy-3-methylglutaryl anhydride was prepared by the method of Louw et al. (11). 3-Methylglutaconyl anhydride was synthesized by the procedure of Adams and Van Duuren (1). 3-Methylcrotonyl-CoA and HMG-CoA were synthesized by the methods of Stadtman (24), which were based on the method of Simon and Shemin (22). The procedure of Lipmann and Tuttle (10) was used for the determination of active acyl groups as their hydroxamic acids, with the volume reduced to 1.0 ml.

The 3-methylglutaconate used for growth studies was synthesized from ethyl isodehydracetate by the method of Feist (4). Hydroxamate standards for paper chromatography were prepared from the corresponding anhydrides, by using the neutral hydroxylamine reagent of Lipmann and Tuttle (10), and were recrystallized from ethanol by the procedure of Sokatch et al. (23).

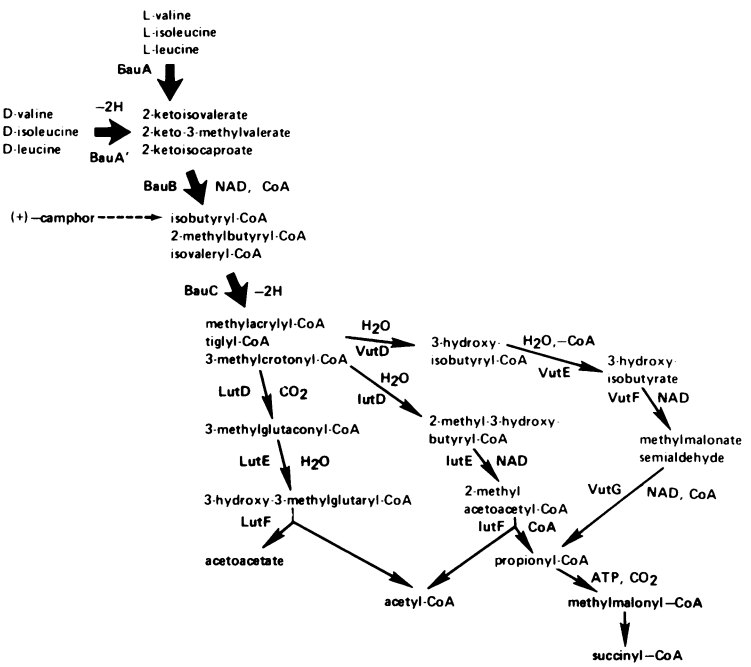

FIG. 2. *Pathway for the metabolism of* D- *and* L-*branched-chain amino acids in pseudomonads. The enzymes proposed to be common are designated by the abbreviation Bau. The reactions they catalyze are represented by wide arrows. Enzymes thought to be unique to leucine, isoleucine, or valine utilization are designated by the symbols Lut, lut, or Vut, respectively. Taken from Martin et al. (15); reproduced with permission of the American Society for Microbiology.*

All other reagents and coupling enzymes were obtained commercially.

Chromatographic methods. For identification of 3-methylglutaconyl-CoA as the product of 3-methylcrotonyl-CoA carboxylation, a large-scale reaction was incubated, and CoA derivatives were converted to their hydroxamates. The reaction mixture contained potassium bicarbonate, 700 μmol; ethylenediaminetetraacetic acid, 8.0 μmol; $MgCl_2$, 80 μmol; adenosine triphosphate (ATP), 3 μmol; glutathione, 1.5 μmol; 3-methylcrotonyl CoA, 8 μM; and protein from cell-free extract, 1.5 mg, all in a final volume of 6.0 ml. After incubation for 30 min at 37 C, the reaction was terminated by the addition of 3.0 ml of neutral hydroxylamine, which was allowed to react 30 min at room temperature. Reaction mixtures with boiled enzyme and synthetic hydroxamates were included as standards.

The hydroxamates were extracted with ethanol, as described by Sokatch et al. (23), and chromatographed for 16 h on Whatman no. 1 filter paper with water-saturated butanol (10). The dried chromatogram was viewed with ultraviolet light and then sprayed with ferric chloride reagent (10).

Assays of enzymatic activity. All specific activities are expressed as nanomoles of product formed per minute per milligram of protein.

Spectrophotometric assays were performed with a Beckman model DU spectrophotometer equipped with a Gilford model 2000 multiple-absorbance recorder. The spectrophotometer was equipped with thermospacers through which water circulated at a temperature of 37 C.

D-Amino acid dehydrogenase (EC 1.4.3.3) was assayed by the method of Norton et al. (16). Branched-chain amino acid transaminase (EC 2.6.1.6) was measured by the assay of Taylor and Jenkins (27). Branched-chain keto acid dehydrogenase was measured by the method of Marshall and Sokatch (13). HMG-CoA lyase (EC 4.1.3.4) was assayed by the method of Stegink and Coon (26). Tiglyl-CoA hydrase and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase were measured by the method of Conrad et al. (2).

3-Methylcrotonyl-CoA carboxylase (EC 6.4.1.6) was assayed by measuring adenosine diphosphate (ADP) produced from adenosine triphosphate (ATP), using a modification of the method of Himes et al. (8). Lactic dehydrogenase, reduced nicotinamide adenine dinucleotide, and bovine serum albumin were omitted from the described assay. The excess of phosphoenolpyruvate and pyruvate kinase catalyzed the regeneration of ATP from ADP and the production of pyruvate. Pyruvate was measured by the method of Friedemann and Haugen (5). Production of pyruvate was linear for 10 min in an assay containing 0.1 to 0.3 mg of protein from cell-free extract.

RESULTS

Growth of P. putida PpG2 with leucine isomers. Toxic effects of L-leucine have been

reported in *P. putida* (15), *Escherichia coli* K-12 (20), and *Hydrogenomonas* H-16 (6). The generation time of *P. putida* PpG2 grown with L-leucine as sole carbon source was five- to sixfold longer than that of cells grown with D-leucine or DL-leucine, respectively (Table 1). Increasing supplemental amounts of D-leucine shortened the generation time. Slow growth in the presence of L-leucine was partially overcome by addition of 0.1-mM amounts of D-valine, L-valine, or 2-ketoisovalerate, and growth in the presence of any one of these supplements resulted in generation times of 8 to 12 h (Table 2). Addition of 0.1 mM L-isoleucine or L-glutamate to L-leucine medium had no effect on the generation time. Because the generation time was shortened by amounts of D-leucine, D- or L-valine, and 2-ketoisovalerate that would not support growth, the slow growth with L-leucine seems to be inhibition rather than a case of a substrate which is metabolized slowly. In any event, work to date has not provided a simple explanation of the effect and, because of the inhibition of growth by L-leucine, DL-leucine was used routinely for growth by *P. putida* in these studies.

Enzymatic activities of leucine catabolism.

TABLE 1. *Relief of L-leucine inhibition by D-leucine in Pseudomonas putida PpG2*

Growth substrate	Growth rate (generation/h)	Generation time (h)
10 mM L-leucine	0.050	20.0
10 mM L-leucine + 0.1 mM D-leucine	0.098	10.2
10 mM L-leucine + 0.5 mM D-leucine	0.142	7.0
10 mM L-leucine + 2.0 mM D-leucine	0.338	3.0
10 mM D-leucine	0.334	3.0
5 mM L-leucine + 5 mM D-leucine	0.347	2.9
10 mM DL-leucine	0.374	2.7

TABLE 2. *Relief of L-leucine inhibition by valine and 2-ketoisovalerate*

Additions to medium with 5 mM L-leucine	Generation time (h)
None	20.4
L-Valine (0.1 mM)	10.8
D-Valine (0.1 mM)	8.3
2-Ketoisovalerate (0.1 mM)	12.0
L-Isoleucine (0.1 mM)	22.2
L-Glutamate (0.1 mM)	25.0

The proposed pathway of DL-leucine catabolism is shown in Fig. 1. Three of these enzymes have been previously demonstrated to be necessary for the oxidation of D- and L-leucine. L-Leucine was deaminated by branched-chain L-amino acid transaminase (17). We found that the pH optimum for L-leucine transamination with extracts of *P. putida* was 9.5 in 0.1 M tris(hydroxymethyl)aminomethane buffer, the same as that previously reported for the purified enzyme from *P. aeruginosa* (17). D-Amino acid dehydrogenase in *P. putida* was induced by D-leucine (13) and during this study was found to have a pH optimum of 8.0 with D-leucine as the substrate. Branched-chain keto acid dehydrogenase was also induced by growth on L-leucine (14). Levels of these enzymes are shown in Table 3.

The carboxylation of 3-methylcrotonyl-CoA which has been shown to occur in extracts of *Pseudomonas oleovorans*, a biotype of *P. putida* (25), has also been demonstrated in extracts of *P. putida* grown on DL-leucine. The product of the reaction, assumed to be 3-methylglutaconyl-CoA, was converted to its hydroxamate which was identified by chromatography (Table 4). The hydroxamate of the product absorbed ultraviolet light and had an R_f corresponding to synthetic 3-methylglutaconyl hydroxamate. Isovaleryl hydroxamate and 3-methyl-3-hydroxyglutaryl hydroxamate do not absorb ultraviolet light and, therefore, would not be detected in this system. Extracts of *P. putida* grown on

TABLE 3. *Levels of leucine catabolic enzymes in P. putida PpG2 grown on leucine, glucose, succinate, and glutamate*

Enzyme	Sp act (nmol/min/mg) when carbon source for growth was:			
	0.3% DL-Leucine	0.3% Glucose	0.3% Succinate	0.3% L-Glutamate
D-Amino acid dehydrogenase	6	0	0	0
Branched-chain amino acid transaminase	154	200	174	178
Branched-chain keto acid dehydrogenase	39	0	0	0
3-Methylcrotonyl-CoA carboxylase	108	0	0	0
3-Hydroxy-3-methylglutaryl-CoA lyase	153	0	0	0

TABLE 4. *Chromotographic identification of the product of 3-methylcrotonyl-CoA carboxylation reaction*

Reaction components	R_f
Complete reaction, active enzyme	0.80, 0.54
Complete reaction, boiled enzyme	0.80
Boiled enzyme plus 3-methylglutaconyl hydroxamate	0.55

DL-leucine were unable to fix CO_2 when tiglyl-CoA or crotonyl-CoA were substituted for 3-methylcrotonyl-CoA in the carboxylase reaction. CO_2 fixation in these studies was followed by our modification of the assay for 3-methylcrotonyl-CoA carboxylase described above.

The hydration of 3-methylglutaconyl-CoA to 3-hydroxy-3-methylglutaryl-CoA was reported by Hilz et al. (7) to occur in a *Mycobacterium* grown on isovalerate. This enzyme was not assayed routinely in this study because of the difficulties associated with preparing the natural substrate for enzyme action which must be in the *trans* configuration.

3-Hydroxy-3-methylglutaryl-CoA was cleaved to equimolar amounts of acetoacetate and acetyl-CoA by extracts of *P. putida* grown on DL-leucine. HMG-CoA lyase has been purified 80-fold, and the reaction has been characterized (L. K. Massey, R. S. Conrad, and J. R. Sokatch, unpublished data).

Inducible enzymes of the leucine catabolic pathway. It is clear from the data in Table 3 that growth of *P. putida* on DL-leucine resulted in the induction of D-amino acid dehydrogenase, branched-chain keto acid dehydrogenase, 3-methylcrotonyl-CoA carboxylase, and HMG-CoA lyase. These enzymes were not detected when *P. putida* was grown on glucose, succinate, or L-glutamate. In contrast, branched-chain amino acid transaminase is a constitutive enzyme (14). 3-Methylcrotonyl-CoA and HMG-CoA lyase were also partially induced when *P. putida* was grown on DL-isoleucine or DL-valine (Table 5), but not on the fatty acid intermediates, crotonate, acetate or 3-hydroxybutyrate.

Induction of the 3-methylcrotonate portion of the pathway. When *P. putida* was grown on isovalerate or 3-methylcrotonate as the carbon source, 3-methylcrotonyl-CoA carboxylase and HMG-CoA lyase were the only two inducible enzymes detected (Table 6). Thus, at least these two enzymes could be induced separately from enzymes in the earlier part of the pathway. In fact, growth on 3-methylcrotonate induced levels of carboxylase and lyase two- to threefold above that observed in *P. putida* grown on isovalerate or DL-leucine. We found that *P. putida* PpG2 did not grow on 3-methylglutaconate or 3-hydroxy-3-methylglutarate, so induction by these compounds could not be determined. None of the four inducible catabolic enzymes could be detected when the organism was grown on acetate or 3-hydroxybutyrate. Hence, back-induction by the products of leucine oxidation, acetate and acetoacetate, was unlikely. Some induction of carboxylase and lyase occurred when *P. putida* was grown on the isoleucine catabolic intermediates, 2-methylbutyrate and tiglate, and on the valine catabolic intermediate, isobutyrate.

Catabolite repression. Repression of synthesis of 3-methylcrotonyl-CoA carboxylase and

TABLE 5. *Levels of leucine-specific catabolic enzymes in P. putida grown on branched-chain amino acids and fatty acid intermediates*

Carbon source (0.3%, wt/vol)	Sp act (nmol/min/mg)	
	3-Methylcrotonyl-CoA carboxylase	3-Hydroxy-3-methylglutaryl-CoA lyase
DL-Leucine	128	205
DL-Isoleucine	87	69
DL-Valine	66	54
Crotonate	0	0
3-Hydroxybutyrate	0	0
Acetate	0	0

TABLE 6. *Inducible leucine catabolic enzymes in P. putida PpG2 grown on branched-chain amino acid catabolic intermediates*

Enzyme	Sp act (nmol/min/mg) when carbon source for growth was:				
	0.3% Isovalerate	0.3% 2-Methylbutyrate	0.3% 3-Methylcrotonate	0.3% Isobutyrate	0.3% Tiglate
D-Amino acid dehydrogenase	0	0	0	0	0
Branched-chain keto acid dehydrogenase	0	0	0	0	0
3-Methylcrotonyl-CoA carboxylase	111	58	308	36	42
3-Hydroxy-3-methylglutaryl-CoA lyase	154	87	391	51	60

HMG-CoA lyase was shown by the addition of glucose or glutamate to cells growing on DL-leucine and synthesizing both of those enzymes (Fig. 3). In a constant volume sample of rapidly growing cells which were synthesizing carboxylase and lyase, the total units of these enzymes increased. The cessation of carboxylase and lyase synthesis due to catabolic repression would cause carboxylase and lyase activity to remain at the level corresponding to total units present at the time repression commenced. However, in these experiments the total carboxylase and lyase activity decreased slightly. Decrease in activity due to the production of an enzyme inhibitor was not likely, since combinations of extracts from fully induced cells and repressed cells had total activity equivalent to the sum of activities of the separate extracts. There was no relief of repression during the period of growth observed in these experiments, however, cultures grown overnight in media containing 0.3% L-glutamate and isoleucine had normal enzyme levels, presumably due to catabolism of the repressor (see Tables 8 and 9).

Growth of P. putida, PpG2, and branched-chain amino acid mutants. Strain PpG736, which was used as a parent in mutant isolation, was unable to utilize valine or isobutyrate as a carbon source (Table 7). However, strain PpG736 could grow normally on isovalerate, leucine, 2-methylbutyrate, and isoleucine, so the genetic block seemed to be specific for

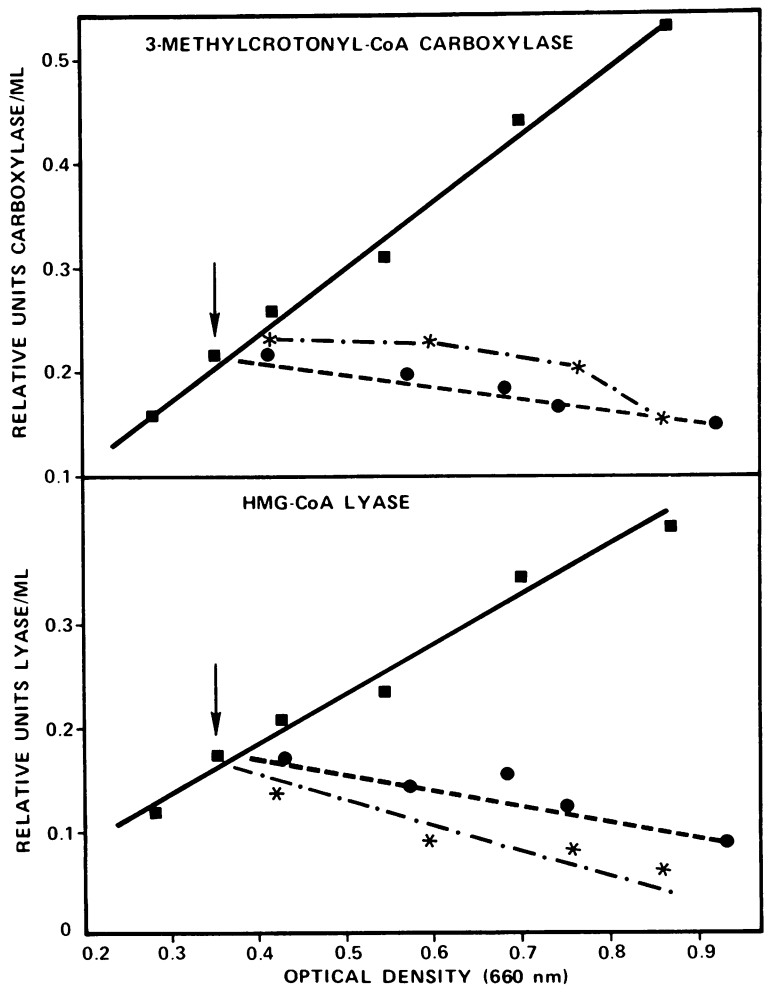

FIG. 3. *Catabolite repression of 3-methylcrotonyl-CoA carboxylase and HMG-CoA lyase by 22 mM glucose (●) or 22 mM L-glutamate (✱) in cultures of P. putida growing on 15 mM DL-leucine (■) as sole source of carbon. Glucose or glutamate was added at the optical density indicated by the arrow. The last sample shown on these figures was taken 2 h and 35 min after addition of glucose and glutamate.*

oxidation of isobutyrate. Several mutants of *P. putida* PpG736 which were unable to utilize isovalerate were isolated after mutagenesis and selection for lack of ability to grow on isovalerate after replica plating from 3-hydroxybutyrate plates. Since acetoacetate was unstable over the period used to determine growth, 3-hydroxybutyrate was used as growth substrate in lieu of acetoacetate to test for ability to degrade acetoacetate. 3-Hydroxybutyrate is known to be dehydrogenated to acetoacetate. Strains unable to utilize isovalerate were also unable to grow on DL-leucine but retained ability to grow on 2-methylbutyrate and DL-isoleucine. Both parent and mutant strains grew on propionate, which was a product of both isoleucine and valine catabolism.

Enzyme induction in mutants. Strain PpM2302, which had lost the ability to grow on isovalerate and leucine, had lowered levels of both 3-methylcrotonyl-CoA carboxylase and hydroxymethylglutaryl-CoA lyase (Table 8). However, the enzymes common to catabolism of all three branched-chain amino acids were normal (Table 9). Strain PpM2302 grown on DL-isoleucine had levels of tiglyl-CoA hydrase and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase comparable to those induced in parent and wild-type strains (Table 9). Thus, the only enzymes that were affected by loss of the ability to grow on isovalerate were enzymes necessary for oxidation of isovalerate.

DISCUSSION

The pathway presented in this paper for the metabolism of DL-leucine in *P. putida* appears to be the same as that known in mammalian tissues. Two enzymes characteristic of leucine catabolism, 3-methylglutaryl-CoA carboxylase and 3-hydroxy-3-methylglutaryl-CoA lyase, were induced when *P. putida* was grown on branched-chain amino acids. These enzymes were also induced by growth on isovalerate, 2-methylbutyrate, and isobutyrate. The inductive effect of valine, isoleucine, and their catabolic intermediates on leucine enzymes is most likely due to the steric similarities of the three branched-chain amino acids and derivatives. Similarly, the valine-specific enzymes, 3-hydroxyisobutyrate dehydrogenase and methylmalonate semialdehyde dehydrogenase, were partially induced by growth on L-isoleucine (14). Conrad et al. (2) found that tiglyl-CoA hydrase and 2-methylbutyryl-CoA dehydrogenase were induced by growth on DL-valine. No straight-chain compounds, such as crotonate or 3-hydroxybutyrate, induced the later enzymes of any of the three branched-chain amino acid pathways, so induction seemed to be restricted to branched-chain compounds derived from leucine, isoleucine, and valine.

TABLE 7. *Growth of Pseudomonas putida wild type and mutants at the expense of various carbon sources related to the metabolism of branched-chain amino acids*

Carbon source (0.3%):	Growth of strains		
	PpG701	PpG736	PpM2302
DL-Valine	+	−	−
Isobutyrate	+	−	−
3-Hydroxyisobutyrate	+	−	−
DL-Isoleucine	+	+	+
2-Methylbutyrate	+	+	+
Propionate	+	+	+
DL-Leucine	+	+	−
Isovalerate	+	+	−
3-Hydroxybutyrate	+	+	+

TABLE 8. *Levels of leucine catabolic enzymes in parent and mutant strains of Pseudomonas putida*

Enzyme	Sp act (nmol/min/mg) when carbon source for growth was:		
	PpG736		PpM2302
	0.3% Isovalerate	0.3% Isovalerate + 0.3% L-glutamate	0.3% Isovalerate + 0.3% L-glutamate
3-Methylcrotonyl-CoA carboxylase	67	66	24
3-Hydroxy-3-methylglutaryl-CoA lyase	268	261	23

TABLE 9. *Levels of D-amino acid dehydrogenase, branched-chain amino acid transaminase, branched-chain keto acid dehydrogenase, tiglyl-CoA hydrase, and 2-methyl-3-hydroxybutyryl-CoA dehydrogenase in parent and mutant strains of P. putida grown on 0.3% DL-isoleucine plus 0.3% L-glutamate*

Enzyme	Sp act (nmol/min/mg)		
	PpG701	PpG736	PpM2302
D-Amino acid dehydrogenase	4.9	5.5	5.0
Branched-chain amino acid transaminase	206	203	210
Branched-chain keto acid dehydrogenase	57	71	66
Tigyl-CoA hydrase	82	129	161
2-Methyl-3-hydroxybutyryl-CoA-dehydrogenase	108	178	183

Although growth on valine and isoleucine induced carboxylase and lyase, these enzymes are not active on any valine or isoleucine catabolic intermediate tested (L. K. Massey, R. S. Conrad, and J. R. Sokatch, unpublished data). Likewise, tiglyl-CoA hydrase, an isoleucine catabolic enzyme, did not hydrate 3-methylglutaconyl-CoA, an unsaturated leucine intermediate (2). In the same report, Conrad et al. found that purified 2-methyl-3-hydroxybutyryl-CoA dehydrogenase was not active with 3-hydroxyisobutyryl-CoA, a valine catabolic intermediate, or with 3-hydroxy-3-methylglutaryl-CoA, a leucine intermediate.

We suspect that the lesion in mutant PpM 2302 is either in the carboxylase or lyase. In addition to the data presented here, we have unpublished observations that the mutant is unable to grow in broth with 3-methylcrotonate although the wild type can. Neither strain can grow with 3-methylcrotonate as the carbon source on solid medium so that the significance of the finding in broth is not completely clear. If it is true that the mutant has lost the ability to grow with 3-methylcrotonate, then the mutation must affect either the carboxylase or lyase rather than acyl-CoA dehydrogenase. Three other mutants selected for inability to use isovalerate also showed reduced levels of carboxylase and lyase similar to the results obtained with PpM 2302 (Table 8).

We believe that the early enzymes of the pathway in *P. putida*, D-amino acid dehydrogenase, branched-chain amino acid transaminase, and branched-chain keto acid dehydrogenase, are common to the metabolism of all three branched-chain amino acids. Data presented in an earlier paper on valine catabolism (14), in this report on leucine catabolism, and in the accompanying paper on isoleucine catabolism (2) support the idea that later enzymes in the respective pathway are unique to the catabolism of each branched-chain amino acid. Therefore, branched-chain amino acid catabolism in *Pseudomonas* is accomplished by diverging catabolic pathways with an initial common segment, followed by specific pathways which feed end products into the tricarboxylic acid cycle.

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