INDUCTION OF SEPARATE CATABOLIC PATHWAYS FOR L- AND D-LYSINE IN PSEUDOMONAS PUTIDA

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Summary: A strain of Pseudomonas putida was found to oxidize L-lysine by an inducible pathway through δ-aminovalerate, and D-lysine via Δ^1-piperideine-2-carboxylate and pipecolic acid. Each pathway is selectively induced by L- or D-lysine and the appropriate intermediates. Lysine racemase is inadequate to permit growth on L-lysine after a block in the L-pathway, but is sufficient to permit cross induction of D-lysine-related enzymes.

In studying the relationship of GSA** dehydrogenase (1,2) to lysine metabolism, we have isolated a number of mutants of Pseudomonas putida blocked in lysine utilization. This report presents evidence for the existence and induction of separate pathways for the utilization of L- and D-lysine and the metabolic interrelationship between the pathways.

MATERIALS AND METHODS

Pseudomonas putida biotype A, ATCC 15070, has been described in detail elsewhere (3). Cells were grown on a mineral salts medium (4) with the substrate indicated as sole carbon source; growth was followed by turbidity measurements using a Klett with a 54 filter. Mutants were prepared by treatment with NG (5) or EMS (6). Negative growth of mutant strains was defined by failure to grow for 48 hours. Lysine racemase was measured under anaerobic conditions by the rate of L-lysine formation from D-lysine (7); GSA dehydrogenase activity as described earlier (1); L-glutamate-AVA transaminase by a coupled reaction using purified α-ketoglutaric semialdehyde dehydrogenase in the presence of AVA, α-ketoglutarate and TPN. Pipecolate oxidase was assayed according to Baginsky and Rodwell (8); the reductase for P2C by the disappearance of TPNH. One activity unit is that amount of enzymes required to produce one micromole of reaction product per minute.

L-Lysine-U-^{14}C and DL-lysine-2-^{14}C were obtained from New England Nuclear

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** Abbreviations used: AOA, aminooxyacetate; AVAM, δ-aminovaleramide; AVA, δ-aminovalerate; EMS, ethyl methane sulfonate; GSA, glutaric semialdehyde; NG, N-methyl-N'-nitro-N-nitrosoguanidine; P2C, Δ^1-piperideine-2-carboxylate; P6C, Δ^1-piperideine-6-carboxylate.

or Calbiochem. Both compounds contained significant impurities which eluted from a Technicon amino acid analyzer at 15, 21, 26 and 41 minutes (see below). D-Lysine-2-^{14}C was prepared from DL-lysine-2-^{14}C by treatment with L-lysine decarboxylase (Worthington) and was separated from cadaverine on Amberlite XE-64 (9). P2C was prepared enzymatically from D-pipecolate by D-amino acid oxidase (Worthington) treatment and was separated on Dowex 50 (H^+) with 0.5 N HCl. P6C was prepared similarly except that the 100,000 x g supernatant solution of L-lysine-grown wild type cell sonic extract was used in place of the oxidase. AVAM was prepared as described previously (10).

Uptake studies followed a published method (11). Metabolite trapping experiments were utilized for studies of the relative fate of labeled L- or D-lysine. Wild type or mutant M-7 cells grown on 0.4% L-glutamate plus 0.4% DL-lysine were washed and suspended in 0.033 M K phosphate buffer, pH 7, to a final density of 1,000 Klett units. Portions (0.8 ml) of this cell suspension were incubated in a final volume of 2 ml with 1.6 μc of D-lysine-2-^{14}C or L-lysine-U-^{14}C (specific radioactivity, 1 mc/mmole) and 48 μmoles of unlabeled L-pipecolate. In wild type cells, 48 μmoles of unlabeled AVA were also added as trapping agent; this was not required for mutant M-7 because of its endogenous accumulation resulting from the lack of enzyme 4 (Fig. 1). The mixture was incubated at 30° for 90 minutes with aeration and the reaction was stopped by addition of acetone to 40% (v/v). After centrifugation the supernatant solution was evaporated to dryness and taken up in 0.5 ml of 0.01 N HCl for analysis on a Technicon amino acid analyzer. Radioactivity was counted in a Packard scintillation counter using a modified Bray's solution (12). The relevant metabolites could be separated from each other by passage through a column (0.9 x 60 cm) of

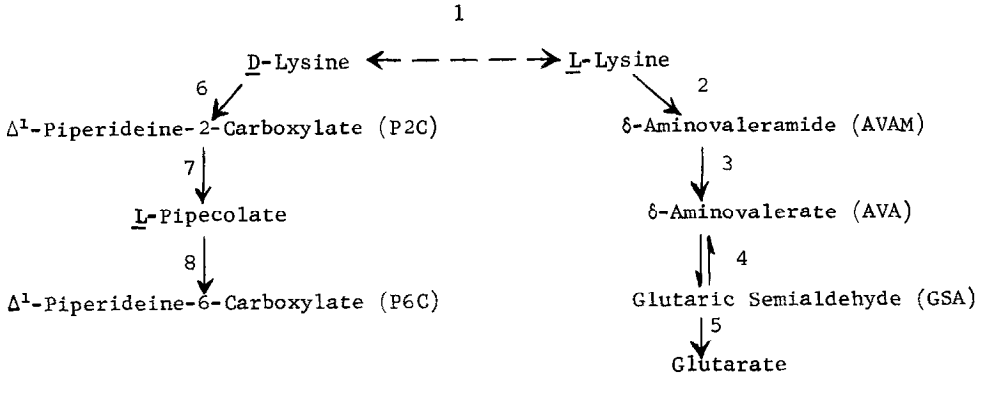

Fig. 1. Lysine Catabolism Proposed in Pseudomonas putida. The enzymes are: 1, lysine racemase; 2, lysine oxygenase; 3, AVAM amidase; 4, glutamate-AVA transaminase; 5, GSA dehydrogenase; 6, not characterized; 7, P2C reductase; 8, pipecolate oxidase. The dotted arrow indicates that the reaction is too slow to support growth.

amino acid analyzer resin (Durrum DC-1A) and elution with Na citrate buffer (Na^+ = 1.6 N), pH 6.35, at 52° at a rate of 35 ml/hr. The following compounds emerged at the times shown: unknown, 12-18 min; AVA, 23-28 min; P6C, 28-34 min; pipecolate, 35-42 min; P2C, 42-48 min; and lysine, 50-58 min.

RESULTS

Growth of Wild Type and Mutant Strains - The wild type strain grows well on all intermediates of the AVA pathway (Fig. 1, right branch), or on D-lysine, on D-, L- or DL-pipecolate, or on P2C, each as sole carbon source. L- or D-Lysine or DL-pipecolate also serve both as carbon and nitrogen sources. Growth on AVAM required two days for full growth, possibly because of limited permeability.

Several mutants, summarized in Table 1, were isolated by the criterion of growth on L-lysine. Mutant M-7 provided key information on the separation of pathways from L- and D-lysine respectively, since it could grow normally on

Table 1. Properties of Mutant Strains of Pseudomonas putida

Strain	Growth Phenotype		Enzyme Defect	Mutagen
M-7	L-lysine	-	Glutamate-AVA transaminase (enzyme 4)	EMS
A-3	"	slow	GSA dehydrogenase (enzyme 5)	NG
I-5, I-8	L- or D-lysine	-	Lysine oxygenase (enzyme 2) and/or AVAM amidase (enzyme 3)	EMS

D-lysine but not at all on L-lysine, due to the presence of a known enzymatic block in the AVA pathway. A-3 grew slowly on L-lysine (presumably because of the presence of low, noninduced levels of catalytically similar dehydrogenases related to catabolic pathways from hydroxyproline and from glucarate (1,2)) but grew on D-lysine at the wild-type rate. Mutants I-5 and I-8 could grow on neither L- nor D-lysine; this behavior has not yet been rationalized enzymatically. All of these mutants, however, grew as well as wild type on L-pipecolate.

These results indicated the requirement of an intact AVA pathway for growth on L-lysine, a conclusion that has not previously been possible from earlier enzyme studies alone. These data also eliminate pipecolate as an intermediate of the L-lysine pathway, a possibility previously suggested by the finding that pipecolate oxidase was efficiently induced by L-lysine (8). Low levels of lysine racemase (about 0.005 unit/mg protein for wild type cells induced with L-lysine) were detected in wild type, M-7, I-5 and I-8. These were evidently not sufficient for conversion of L-lysine to D-lysine to support

growth, as indicated by the growth behavior of M-7 cells. It is notable that levels of the racemase in our cells were considerably lower than those reported by Ichihara et al (13) for another Pseudomonas strain, in which D-lysine was assumed to be converted to L-lysine for further degradation.

Labeling Intermediates from L- and D-Lysine - Mutant M-7 cells grow on D-lysine or pipecolate but not on L-lysine, suggesting an independent pathway of D-lysine utilization through pipecolate. With ^{14}C-D-lysine and ^{14}C-L-lysine we demonstrated the predominant utilization of D-lysine through pipecolate and of L-lysine through AVA (Table 2). When D-lysine-2-^{14}C was substrate, labeled pipecolate was the major product showing about 7-fold higher formation than when L-lysine-U-^{14}C was substrate. The labeled pipecolate was identified as the L-isomer by treating it with D-amino acid oxidase in the presence of unlabeled D- and L-pipecolate; the P2C formed by this treatment was unlabeled. Labeled P6C was also found in significant quantity, due to the presence of pipecolate oxidase activity in the induced cells.

Table 2. Products of Lysine Utilization in Wild Type and Mutant M-7 of Pseudomonas putida

	Wild Type		Mutant M-7	
	D-lysine	L-lysine	D-lysine	L-lysine
	cpm(x10^{-3})			
Lysine added	2560	2560	2560	2560
Pooled peaks recovered:				
Unknown	57	49	60	59
AVA	trace	55	trace	32
P6C	39	trace	45	trace
Pipecolate	186	33	205	28
Lysine	1233	1314	881	1027
Total	1515	1451	1191	1146

The labeled substrates were D-lysine-2-^{14}C and L-lysine-U-^{14}C. The AVA and P6C peaks slightly overlapped. A trace of activity represents less than 2000 cpm.

When L-lysine-U-^{14}C was the incubated substrate, formation of labeled pipecolate was markedly reduced while another labeled intermediate, AVA, appeared. More than 50% of the unlabeled L-pipecolate in the trapping pool was recovered after incubation as estimated by the method of Piez et al (14). The difference in pipecolate labeling from L- or D-lysine is not due to uptake difference of these two isomers, since the rate of uptake of both isomers was quite similar in both strains (Chang and Adams, unpublished results).

Table 3. Induction of Pipecolate Oxidase and GSA Dehydrogenase.

Strain	Growth Substrate	Pipecolate Oxidase	GSA Dehydrogenase
		units/mg protein	
Wild type	L-Glutamate	0.21	0.04
	" + L-Lysine	11.0	0.14
	" + D-Lysine	14.0	0.1
	" + AVAM	0.11	0.14
	" + AVA	0.13	0.25
	" + P2C	6.7	0.21
	DL-Pipecolate	4.5	0.11
Mutant M-7	L-Glutamate	0.23	0.01
	" + L-Lysine	4.34	0.60
	" + D-Lysine	6.75	0.22
	" + AVA	0.71	0.73
	DL-Pipecolate	7.3	0.08

The specific activities reported are the average of at least two experimental determinations. All growth substrates were added at 0.2% each except P2C which was 0.03%. Cultures were sampled during exponential growth at 24 hours after inoculation.

Enzyme Induction in the L- and D-Lysine Pathways - Table 3 shows the levels of pipecolate oxidase and GSA dehydrogenase in extracts of mutant M-7 and wild type cells grown on various substrates. In mutant M-7 L-glutamate was required as growth substrate in addition to the inducers. Controls with wild type cells showed that glutamate had no appreciable repressive or inhibitory effect on enzymes induced by lysine or its intermediates. From these data and many other such observations, it appears that L-lysine induces higher levels of GSA dehydrogenase than does D-lysine and that D-lysine is consistently a better inducer of pipecolate oxidase than is L-lysine. Of all the intermediates in the AVA pathway, the best inducer of GSA dehydrogenase is AVA. This is demonstrated directly (in both mutant and wild type strains) as well as by the greater induction effect on lysine in the M-7 mutant, in which AVA should accumulate before the blocked step. Neither AVA nor AVAM, consistent inducers of GSA dehydrogenase, induced pipecolate oxidase. In a few experiments (limited by the availability of this substrate), P2C appeared to induce GSA dehydrogenase as well as pipecolate oxidase. In summary, the only consistent inducer of both enzymes was lysine itself. It is also noteworthy (data not shown here) that both D- and L-lysine are inducers of P2C reductase (7- to 8-fold) and glutamate-AVA transaminase (18- and 28-fold, respectively). The latter finding contrasts with the non-inducibility of glutamate-AVA transaminase reported by Suda et al (16) in their strain of Pseudomonas.

That D-lysine has a special relation to pipecolate oxidase was suggested

by the difference in response to L- and D-lysine by two mutants I-5 and I-8 (Table 1). In I-5, only D-lysine could produce distinct induction of pipecolate oxidase, while in I-8 both L- and D-lysine were approximately equivalent inducers. A mutant lacking lysine racemase might provide definitive evidence for the relative role of L-lysine and D-lysine as inducers either of the AVA or the pipecolate pathway, but many attempts to obtain such a mutant failed.

As an alternative, AOA was used as a probable inhibitor of the lysine racemase. This compound, a carbonyl reagent, probably acts as a binding agent of the racemase cofactor, pyridoxal phosphate. When cells grown on L-glutamate were exposed to L- or D-lysine in the presence of AOA, only D-lysine was capable of inducing pipecolate oxidase (Fig. 2). Time-studies of pipecolate oxidase levels showed that even in the absence of AOA, the enzyme appeared considerably more slowly in response to L-lysine than to D-lysine (Fig. 2). A simple interpretation is that D-lysine is the specific inducer of pipecolate oxidase and that L-lysine can induce the enzyme only by way of its enzymatic racemization to the D-antipode.

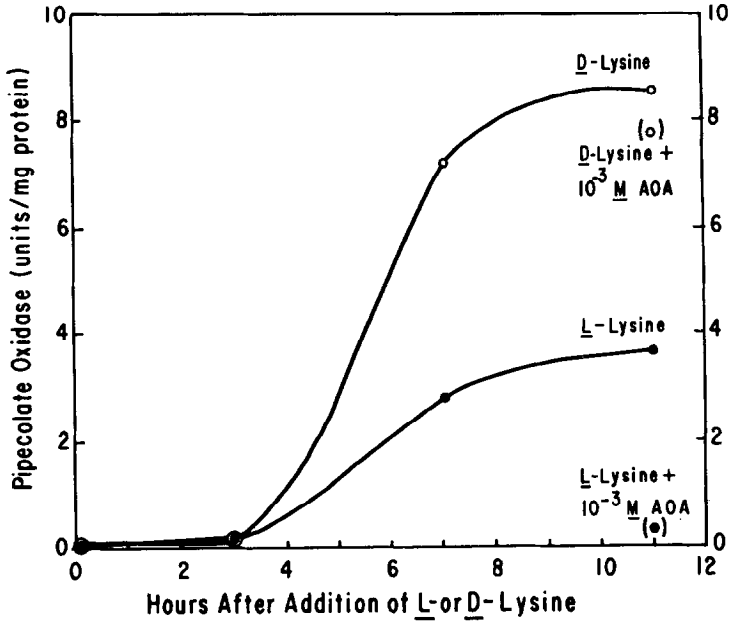

Fig. 2. Pipecolate Oxidase Induction by D- and L-Lysine. Wild type cells were originally grown on 0.2% L-glutamate. Inducer concentration was 0.2%.

DISCUSSION

The data presented lead us to conclude that separate inducible pathways for L-lysine and D-lysine are present in our Pseudomonas strain. The growth data suggest that the level of lysine racemase in our Pseudomonas strain is

much lower than that described in another Pseudomonas strain (13) and is insufficient to permit the M-7 cell to convert L-lysine to D-lysine at a rate sufficient for growth. Other data, however, suggest that this low level of lysine racemase is responsible for the induction of pipecolate oxidase by L-lysine, through the conversion of the latter to D-lysine.

While this report was in preparation, somewhat similar conclusions concerning the two pathways from D- and L-lysine were reported by Miller and Rodwell (15) for another strain of Pseudomonas putida. Our results, perhaps because of a lower level of lysine racemase in wild type cells, and with the aid of a mutant blocked in the AVA pathway, indicate the divergent pathways more clearly.

A further result of this study is that P2C is an intermediate in the catabolism of D-lysine as shown by the experiments with ^{14}C-D-lysine. An enzyme catalyzing TPNH-linked reduction of P2C to L-pipecolate was also detected in cell extracts and was induced by growth of cells on D- or L-lysine. Similarly, P2C itself induced both pipecolate oxidase and GSA dehydrogenase.

In animal tissues, L-lysine appears to be degraded via saccharopine (17, 18), but D-lysine is primarily converted to pipecolate (19). Our present findings support the view that a D-lysine → L-pipecolate pathway is a general one, found in bacteria as well as mammals. Earlier reports of an enzyme catalyzing P2C → L-pipecolate in rat liver and other mammalian tissues, as well as in microorganisms and plants (21, 21), are consistent with this conclusion. In our strain of Pseudomonas, the two pathways appear to be physiologically interrupted at the racemase step between L-lysine and D-lysine, yet each pathway is induced by intermediates of the other pathway. From this we conclude that lysine racemase is inadequate to permit growth on L-lysine after a block in the AVA pathway, but is sufficient to permit cross-induction of D-lysine-related enzymes (e.g., pipecolate oxidase) by L-lysine. This limited flow of metabolites between the two pathways may explain the survial in cells of regulatory relationships even when the pathways involved may no longer be physiologically viable, as suggest by Cánovas et al (22).

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