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Metabolism of Allylglycine and *cis*-Crotylglycine by *Pseudomonas putida* (*arvilla*) mt-2 Harboring a TOL Plasmid

DANIEL A. KUNZ,*† DOUGLAS W. RIBBONS,[1]‡ AND PETER J. CHAPMAN[2]

Department of Biochemistry, School of Medicine, University of Miami, Miami, Florida 33101,[1] and Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108[2]

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Spontaneous mutants which acquired the ability to utilize D-allylglycine (D-2-amino-4-pentenoic acid) and DL-*cis*-crotylglycine (DL-2-amino-*cis*-4-hexenoic acid) but not L-allylglycine or DL-*trans*-crotylglycine could be readily isolated from *Pseudomonas putida* mt-2 (PaM1). Derivative strains of PaM1 putatively cured of the TOL (pWWO) plasmid were incapable of forming mutants able to utilize the amino acids for growth; however, this ability could be regained by conjugative transfer of the TOL (pWWO) plasmid from a wild-type strain of mt-2 or of the TOL (pDK1) plasmid from a related strain of *P. putida* (HS1), into cured recipients. DL-Allylglycine-grown cells of one spontaneous mutant (PaM1000) extensively oxidized DL-allylglycine and DL-*cis*-crotylglycine, whereas only a limited oxidation was observed toward L-allylglycine and DL-*trans*-crotylglycine. Cell extracts prepared from PaM1000 cells contained high levels of 2-keto-4-hydroxyvalerate aldolase and 2-keto-4-pentenoic acid hydratase, the latter enzyme showing higher activity toward 2-keto-*cis*-4-hexenoic acid than toward the *trans* isomer. Levels of other enzymes of the TOL degradative pathway, including toluate oxidase, catechol-2,3-oxygenase, 2-hydroxymuconic semialdehyde hydrolase, and 2-hydroxymuconic semialdehyde dehydrogenase, were also found to be elevated after growth on allylglycine. Whole cells of a putative cured strain, PaM3, accumulated 2-keto-4-pentenoic acid from D-allylglycine, which was shown to be rapidly degraded by cell extracts of PaM1000 grown on DL-allylglycine. These same cell extracts were also capable of catalyzing the dehydrogenation of D- but not L-allylglycine and were further found to metabolize the amino acid completely to pyruvate and acetaldehyde. Differential centrifugation of crude cell extracts localized D-allylglycine dehydrogenase activity to membrane fractions. The results are consistent with a catabolic pathway for D-allylglycine and DL-*cis*-crotylglycine involving the corresponding keto-enoic acids as intermediates, the further metabolism of which is effected by the action of TOL plasmid-encoded enzymes.

Pseudomonas putida (*arvilla*) mt-2 and other pseudomonads degrade toluene, *m*- and *p*-xylene, and related hydrocarbons by using TOL plasmid-encoded enzymes (Fig. 1) (11, 17, 36-38). The catabolic pathways used for degradation of the hydrocarbons involve initial oxidative steps to give aromatic acids followed by ring dioxygenation, forming the corresponding catechols (8, 17, 38). Thus, catechol, 3-methylcatechol, and 4-methylcatechol are formed from toluene, *m*-xylene, and *p*-xylene, respectively. The further metabolism of catechol and its methyl homologs then occurs via the *meta*-fission pathway, generating pyruvate as a tricarboxylic acid cycle precursor. This pathway is utilized for the degradation of a variety of other aromatic structures such as benzene (14; D. A. Kunz and P. J. Chapman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, Q85, p. 275), naphthalene (3, 9), salicylate (4), phenol (7), *o*- and *m*-cresol (2, 27), and *p*-cresol (2), all of which are metabolized through catechol or its methyl homologs. Beyond ring cleavage, the *meta* pathway diverges into separate branches known as the oxidative and hydrolytic branches. For example, 2-hydroxymuconic semialdehyde, generated

† Present address: Central Research and Development Department, E. I. du Pont de Nemours & Co., Inc., Wilmington, DE 19898.

‡ Present address: Department of Biochemistry and Soil Science, University College of North Wales, Bangor, Gwynedd LL57 2UW, Wales, United Kingdom.

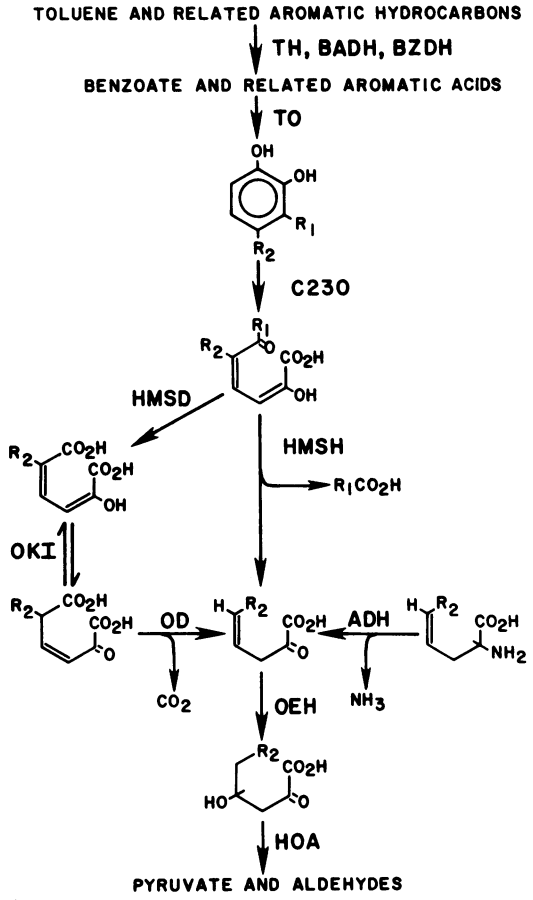

FIG. 1. *TOL plasmid-encoded pathways for the degradation of aromatic substrates and for the metabolism of the Δ^4-unsaturated amino acids allylglycine and cis-crotylglycine. For toluene, R1 = H, R2 = H; for m-xylene, R1 = CH_3, R2 = H; for p-xylene, R1 = H, R2 = CH_3; for 3-ethyltoluene, R1 = CH_2CH_3, R2 = H; for pseudocumene (1,2-4-trimethylbenzene), R1 = CH_3, R2 = CH_3; for allylglycine, R2 = H; for cis-crotylglycine, R2 = CH_3. Enzyme abbreviations not given in text: TH, toluene hydroxylase; BADH, benzyl alcohol dehydrogenase; BZDH, benzaldehyde dehydrogenase; TO, toluate oxidase; OKI, 4-oxalocrotonate ketoisomerase; OD, oxalcrotonate decarboxylase; ADH, allylglycine-dependent amino acid dehydrogenase.*

from catechol, can be metabolized either via NAD^+-dependent dehydrogenation to 4-oxalocrotonate or, alternatively, by direct enzymatic hydrolysis yielding formate and 2-keto-4-pentenoate (2-oxopent-4-enoate) (1, 3, 7, 30, 31). In contrast, cleavage of 3-methylcatechol results in the formation of the methylketone, 2-hydroxy-6-oxo-2,4-heptadienoate, the further metabolism of which occurs exclusively via the hydrolytic branch, giving rise to acetate and 2-keto-4-pentenoate. The oxidative route predominates over the hydrolytic branch for ring fission products generated from catechol and 4-methylcatechol (3, 31); however, both branches converge to give 2-keto-4-pentenoate when catechol and 3-methylcatechol are degraded or to give 2-keto-*cis*-4-hexenoic acid in the case of 4-methylcatechol (1). The next step in the pathway involves hydration furnishing 2-keto-4-hydroxyvalerate (4-hydroxy-2-oxopentenoate) or, in the case of 2-keto-*cis*-4-hexenoic acid, 2-keto-4-hydroxycaproic acid. The hydroxyoxo acids then undergo aldol fission to pyruvate, the former yielding also acetaldehyde and the latter yielding propionaldehyde (Fig. 1) (5, 7).

Because of the unavailability and general instability of intermediates of the *meta*-fission pathway, it has not been possible to test whether intermediates beyond catechol can serve as growth substrates. This would be advantageous for further studies on the genetics and biochemistry of this reaction sequence. We therefore set out to determine whether certain Δ^4-unsaturated amino acids such as allylglycine (2-amino-4-pentenoic acid) and *cis*-crotylglycine (2-amino-*cis*-4-hexenoic acid), structural analogs of 2-keto-4-pentenoic acid and 2-keto-*cis*-4-hexenoic acid, respectively, might substitute as growth substrates for strains carrying TOL plasmids and thus capable of expressing the *meta* pathway.

The catabolism of allylglycine and *cis*-crotylglycine by bacteria has not previously been reported. Skinner and co-workers (33) showed that *cis*-crotylglycine served as a methionine antagonist in *Escherichia coli* and thus inhibited growth; however, they did not investigate the metabolism of this compound. Several investigations on the mammalian metabolism of allylglycine have been conducted (15, 20, 25). Interest in allylglycine metabolism by mammalian tissues, particularly brain, has been generated by the known tendency of this compound to induce seizures when administered to higher organisms (15). The active species has been proposed to be 2-keto-4-pentenoic acid (25); however, because of its instability, direct chemical evidence for its involvement in inducing convulsive seizures has not been obtained. Collinsworth et al. (5) reported that snake venom L-amino acid oxidase could be used to generate 2-keto-4-pentenoate from allylglycine, which was used as a substrate for 2-keto-4-pentenoate (vinylpyruvate) hydratase (OEH) purified from *Pseudomonas* U. Marcotte and Walsh (20) showed that several products, including 2-keto-4-pentenoate, were formed from the action of snake venom L-amino acid oxidase activity on L-allylglycine, the product formed being highly dependent on incuba-

tion conditions. They further showed that 2-keto-4-pentenoate was in rapid equilibrium with 2-hydroxy-2,4-pentadienoate, both of which reacted in subsequent slower steps to yield the more stable *trans*-2-keto-3-pentenoate.

In this paper, we show that D-allylglycine and DL-*cis*-crotylglycine but not DL-*trans*-crotylglycine (DL-2-amino-*trans*-4-hexenoic acid) can serve as growth substrates for *P. putida* mt-2 (PaM1) carrying a TOL plasmid but not for strains having apparently been cured of this plasmid. It is further shown that the ability to grow with these amino acids is facilitated through the action of TOL plasmid-encoded enzymes. Utilization also requires a D-specific amino acid dehydrogenase reponsible for conversion of the amino acids to the corresponding keto-enoic acids (Fig. 1). The latter ability does not appear to depend on the presence of a TOL plasmid. A preliminary account of some of this work has been reported (D. A. Kunz and P. J. Chapman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, K72, p. 138).

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. Two independent cultures of *P. putida* (*arvilla*) mt-2, designated PaM1 and PaW1, respectively, were obtained from G. D. Hegeman, Department of Microbiology, Indiana University, and P. A. Williams, University College of North Wales, U.K. Strain PaM1000, used extensively for various experiments described here, was obtained as a spontaneous mutant of PaM1 after streaking cells to plates containing DL-allylglycine as the sole carbon source. Table 1 describes other derivatives of *P. putida* mt-2 as well as other strains used in this investigation.

Maintenance and cultivation conditions were conducted as described in previous publications (17, 18, 27). Growth tests were performed on minimal agar plates with water-soluble substrates supplied at 5 mM, whereas volatile substrates were supplied as the vapor (13). Where allylglycine and crotylglycine served as carbon sources, these were filter sterilized before use, whereas other water-soluble substrates were generally autoclaved. Cells grown on allylglycine were obtained by supplying DL-allylglycine at 10 mM and harvesting after 24 to 36 h of incubation on a rotary shaker at 30°C. Alternatively, to increase growth yields, cells

TABLE 1. *List of strains*

Strain designation	Relevant phenotype[a]	Relevant genotype[b]	Parent strain	Reference
PaM1 (*P. putida* mt-2, Minnesota)	Ben^+ Tal^+ Tln^+ Xyl^+ Alg^m	wt/TOL (pWWO)		17
PaM1000	Ben^+ Tal^+ Tln^+ Xyl^+ Alg^+	*alg-1*/TOL	PaM1	This work
PaM3	Ben^+ Tal^- Tln^- Xyl^- Alg^-	TOL^{del}	PaM1	17
PaM300	Ben^+ Tal^+ Tln^+ Xyl^+ Alg^m	wt/TOL (pWWO)	PaW15 × PaM3	17
PaM310	Ben^+ Tal^+ Tln^+ Xyl^+ Alg^m	wt/TOL (pDK1)	PpC14 × PaM3	This work
PaW1 (*P. putida* mt-2, Wales)	Ben^+ Tal^+ Tln^+ Xyl^+ Alg^+	wt/TOL (pWWO)		17, 36
PaW15	Ben^+ Tal^+ Tln^+ Xyl^+ Alg^+ Leu^-	*leu-1*/TOL (pWWO)	PaW1	36
PpC1 (*P. putida* HS1)	Ben^+ Tal^+ Tln^+ Xyl^+ Alg^-	wt/TOL (pDK1)		18
PpCC1	Ben^+ Tal^- Tln^- Xyl^- Alg^-	TOL^{del}	PpC1	18
PpC14	Ben^+ Tal^+ Tln^+ Xyl^+ Alg^- Leu^- $Ileu^-$ Val^- His^-	*leu-1 ileu-1 val-1 his-1*/TOL	PpC1	18
PpC800	Ben^+ Tal^+ Tln^+ Xyl^+ Alg^-	wt/TOL (pWWO)	PaW15 × PpCC1	This work
Pseudomonas sp. strain Pxy	Ben^+ Tal^+ Tln^+ Xyl^+ Alg^m	wt/XYL		12
P. putida ST3	Ben^+ Tal^+ Tln^+ Xyl^+ Alg^-			This work

[a] Phenotype abbreviations: Ben, benzoate; Tal, *m*- and *p*-toluate, 3,4-dimethylbenzoate, 3-ethylbenzoate; Tln, toluene; Xyl, *m*- and *p*-xylene, pseudocumene, 3-ethyltoluene; Alg^m, spontaneous mutants capable of growth on allylglycine observed; Leu, leucine; Ileu, isoleucine; Val, valine; His, histidine.

[b] Genotype abbreviations: wt, wild type; *alg-1*, allylglycine-acquisitive mutant; TOL^{del}, TOL plasmid deletion; pWWO, TOL plasmid from *P. putida* mt-2; pDK1, TOL plasmid from *P. putida* HS1; XYL, TOL-type plasmid from *Pseudomonas* Pxy.

were grown on 10 mM succinate plus 5 mM DL-allylglycine. Growth of cells on other carbon sources and cell-harvesting procedures were performed according to published methods (17, 18).

Genetic crosses between strains of *P. putida* were conducted as described by Kunz and Chapman (17). Exconjugants were selected on agar plates containing *m*-toluate as the sole carbon source and were purified at least twice on selection media before scoring for the ability to utilize allylglycine and aromatic substrates.

Measurement of oxidation rates. Oxidation rates were measured by oxygen consumption both manometrically with constant-volume manometers (Braun, Melsungen, Germany) and polarographically with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) at 30°C.

Preparation of cell extracts and enzyme assays. Cell extracts were prepared either by passing a frozen-cell paste through a Hughes press (16) and suspending the broken-cell contents in 2 volumes of sodium potassium phosphate buffer (pH 7.0) or by passing a cell suspension (1 g [wet weight] of cells per 2 ml of buffer) through a French press (American Instrument Co., Silver Spring, Md.) at 5 to 10°C. One milligram of DNase (Sigma Chemical Co., St. Louis, Mo.) was added to reduce the viscosity, and the suspension was centrifuged at 20,000 × *g* for 25 min at 4°C to give a supernatant referred to as the crude extract. Fractionation of cell extracts into soluble and particulate fractions was accomplished by centrifuging crude extracts at 100,000 × *g* for 1 h at 4°C.

The following enzymes were assayed according to published procedures: catechol-2,3-oxygenase (C23O) (EC 1.13.11.2; catechol:oxygen 2,3-oxido-reductase) (17, 30); 2-hydroxymuconic semialdehyde hydrolase (HMSH) (18, 36); 2-hydroxymuconic semialdehyde dehydrogenase (HMSD) (31); OEH (5); and 2-keto-4-hydroxyvalerate aldolase (HOA) (30). The substrate for OEH was routinely prepared by oxidizing solutions of DL-allylglycine with snake venom L-amino acid oxidase (Sigma) as described by Collinsworth et al. (5). 2-Keto-4-hydroxyvalerate was prepared by alkaline hydrolysis of 4-methyl-2-oxobutyrolactone (7), prepared as described by Rossi and Schinz (29).

Measurements of D-amino acid dehydrogenase activity were made by following the reduction of dichlorophenolindophenol (DCPIP) at 600 nm as described by Norton et al. (22). Reaction mixtures contained, in 3 ml: 83 mM Tris (pH 7.6), 67 μM DCPIP, 0.33 mM NaCN, 2.5 mM allylglycine or other amino acid, and cell extract (0.5 to 1.0 mg of protein). After the background reduction rate was monitored for 6 min, 0.3 ml of amino acid was added and the stimulation in DCPIP reduction was recorded. Specific activities were calculated by using a molar extinction coefficient for DCPIP of $2.1 \times 10^4\ M^{-1}$ (22).

Analytical procedures. Spectrophotometric determinations were made with either a Unicam SP1800 or a Perkin-Elmer model 124 UV-visible recording spectrophotometer. Protein was estimated by the method of Lowry et al. (19), using bovine serum albumin as a standard. Acetaldehyde and pyruvate were detected by thin-layer chromatography of their 2,4-dinitrophenylhydrazones on silica gel plates (Eastman chromatogram sheets no. 13181; Eastman Organic Chemical Division, Eastman Kodak Co., Rochester, N. Y.) with a solvent system containing benzene-tetrahydrofuran-acetic acid (60:34:4) (10). Pyruvate and acetaldehyde were also determined with lactate (rabbit muscle) and alcohol dehydrogenase (Sigma), respectively (34).

Chemicals. DL-, D-, and L-allylglycine were purchased from Sigma. DL-*cis*-Crotylglycine (DL-2-amino-*cis*-4-hexenoic acid) and DL-*trans*-crotylglycine (DL-2-amino-*trans*-4-hexenoic acid) were graciously provided by Paul Talalay, The Johns Hopkins University, Baltimore, Md. Identification of the *cis* and *trans* isomers of crotylglycine was confirmed by comparing R_f values after descending paper chromatography with values for separate samples provided by W. Shive, University of Texas, Austin, and with previously published values (32). The solvent system was *n*-propanol–water (4:1), and compounds were revealed with ninhydrin reagent (0.2% ninhydrin in acetone containing 2,6-lutidine). All other chemicals were of the highest chemical purity available and were obtained as previously described (17, 18).

RESULTS

Genetic role of TOL plasmids in allylglycine utilization. Two independent cultures of *P. putida* mt-2, designated PaM1 and PaW1, were capable of growth with DL-allylgycine and DL-*cis*-crotylglycine but not with DL-*trans*-crotylglycine (Table 2). It was further observed that whereas PaW1 was capable of uniform growth on these amino acids after 48 h of incubation, growth of PaM1 took place only after the selection of spontaneous mutants that appeared at a frequency of approximately 10^{-4}. When one such mutant of PaM1 (PaM1000) was tested for its ability to utilize the separate enantiomers of allylglycine, only the D-isomer was found to support growth (Table 2). Similar results were obtained with strain PaW1. In addition, spontaneous mutants of PaM1 could be observed on agar plates supplied either D-allylglycine or DL-*cis*-crotylglycine but not L-allylglycine or DL-*trans*-crotylglycine. The formation of spontaneous mutants able to utilize these amino acids by PaM1 was paralleled by the ability of PaW1 to utilize the same range of substrates but without prior mutation.

Derivatives of PaM1 apparently deleted of their TOL degradative functions as evidenced by inability to grow with toluene, *m*- and *p*-xylene, pseudocumene (1,2,4-trimethylbenzene), and 3-ethyltoluene, or their oxidation products, were also unable to grow on allylglycine or *cis*-crotylglycine (e.g., PaM3) (Table 2). These results suggested that utilization of the amino acids was dependent on the TOL (pWWO) plasmid. This was further confirmed by showing

TABLE 2. *Growth properties of strains of P. putida*

Growth substrate	Growth of given strain[a]									
	PaM1	PaW1	PaM1000	PaM3	PaM300	PaM310	PpC1	PpC800	Pxy	ST3
DL-Allylglycine	m	+	+	−	m	m	−	−	m	−
D-Allylglycine	m	+	+	−	ND	ND	ND	ND	ND	ND
L-Allylglycine	−	−	−	−	ND	ND	ND	ND	ND	ND
DL-*cis*-Crotylglycine	m	+	+	−	m	m	−	−	ND	ND
DL-*trans*-Crotylglycine	−	−	−	−	−	−	−	−	ND	ND
Toluene	+	+	+	−	+	+	+	+	+	+
Benzoate	+	+	+	+	+	+	+	+	+	+
m-Xylene	+	+	+	−	+	+	+	+	+	+
m-Toluate	+	+	+	−	+	+	+	+	+	+
p-Xylene	+	+	+	−	+	+	+	+	+	+
p-Toluate	+	+	+	−	+	+	+	+	+	+
1,2,4-Trimethylbenzene	+	+	+	−	+	+	+	+	+	+
3-Ethyltoluene	+	+	+	−	+	+	+	+	+	+

[a] +, Good growth; −, no growth; m, acquisitive mutants capable of growth observed; ND, not determined.

that exconjugants obtained by mating a wild-type strain of *P. putida* mt-2 (PaW15) with an apparent cured derivative (PaM3) simultaneously regained the ability to grow on allylglycine, *cis*-crotylglycine, and the aromatic substrates (e.g., PaM300) (Table 2).

When a separate TOL-carrying strain of *P. putida* (HS1) previously shown to resemble *P. putida* mt-2 in its ability to degrade toluene, the xylenes, and related hydrocarbons (18) was tested for the ability to utilize allylglycine and *cis*-crotylglycine, it was found not to do so (e.g., PpC1) (Table 2). However, exconjugants obtained after mating strain HS1 with the putative cured derivative of strain mt-2 (PaM3) regained the ability to grow with DL-allylglycine and DL-*cis*-crotylglycine (e.g., PaM310), whereas exconjugants obtained in the converse genetic mating (e.g., PpC800) were unable to do so. These results suggested that besides a dependence on TOL plasmid-specified functions, an additional host cell factor apparently present in strain mt-2 only was necessary for growth on the amino acids. Two other strains of *P. putida* capable of growth on the same range of aromatic substrates degraded by strains mt-2 and HS1 were also tested for their ability to grow with allylglycine and *cis*-crotylglycine. One of these, *Pseudomonas* Pxy, reported to carry a nonconjugative TOL-type plasmid designated XYL (12), formed mutants able to utilize allylglycine at a frequency similar to that observed for PaM1 (10^{-4}), whereas the other strain, ST3, was unable to utilize or give rise to mutants capable of growth with any of the amino acids (Table 2).

Whole-cell oxidations. Washed-cell suspensions of strain PaM1000 grown on DL-allylglycine rapidly oxidized DL-allylglycine, DL-*cis*-crotylglycine, and DL-*trans*-crotylglycine, whereas L-allylglycine was oxidized at a low rate (Fig. 2; Table 3). Although DL-*trans*-crotylglycine underwent rapid initial oxidation (Fig. 2), the extent of oxidation (0.9 ml of O_2 per mol of amino acid) fell far short of that observed for DL-*cis*-crotylglycine (3 mol of O_2 per mol of amino acid) supplied at the same concentration. Interestingly enough, the aromatic substrates benzoate, *m*-toluate, catechol, and 3-methylcatechol were rapidly oxidized under these conditions. Succinate-grown cells of PaM1000 showed little activity toward DL-allylglycine; however, catechol and 3-methylcatechol were rapidly oxidized. These results suggested that induction was required for allylglycine oxidation, whereas oxidation of the aromatic substrates by this strain was not. It should be noted that no appreciable oxidation of benzoate was observed by succinate-grown cells of PaM1000, in contrast to significant rates observed with catechol and 3-methylcatechol. *m*-Toluate-grown cells were well induced for oxidation of benzoate and catechol but not for DL-allylglycine, which was oxidized at low rates similar to those observed for cells grown on succinate (Table 3).

Enzyme activities in cell extracts. Cell extracts prepared from DL-allylglycine-grown cells of PaM1000 contained elevated levels of OEH and HOA as compared with the levels obtained after growth on succinate (Table 4). It was further observed that crude extracts containing elevated levels of OEH were also able to catalyze the hydration of 2-keto-*cis*-4-hexenoic acid, although the rate was only half that obtained with 2-keto-4-pentenoate (data not shown). 2-keto-*trans*-4-hexenoic acid served as a poor substrate for this activity, being attacked at only about 9% the rate observed for 2-keto-4-pentenoate. Table 4 shows that other *meta*-pathway enzymes, including C230, HMSH, and HMSD, were also present at high levels after

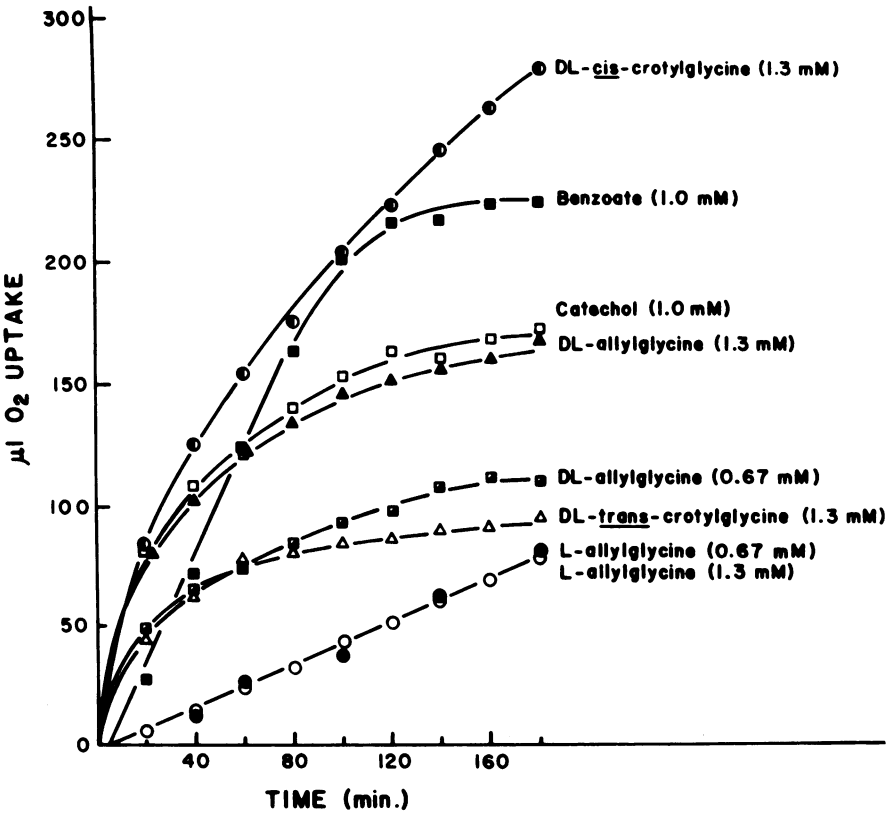

FIG. 2. *Oxidation of Δ^4-unsaturated amino acids and aromatic compounds by allylglycine-grown cells of PaM1000. Each Warburg flask contained: 20 mM phosphate buffer, pH 7.0, containing 140 μg of chloramphenicol (1 ml); cell suspension (1 ml, 20 mg [wet weight]); 0.67 or 1.3 mM substrate (0.2 or 0.4 ml) as indicated; and water to 2.8 ml. The center well contained 20% KOH (0.2 ml). Temperature, 30°C. Results are corrected for endogenous rates measured in the absence of substrate.*

TABLE 3. *Rates of oxidation by intact cells of PaM1000*

Assay substrate	μl of O_2 consumed h^{-1} mg^{-1} (dry wt) after growth with[a]:		
	DL-Allylglycine	*m*-Toluate	Succinate
DL-Allylglycine	75	15	10
L-Allylglycine	7	ND	ND
DL-*cis*-Crotylglycine	62	ND	ND
DL-*trans*-Crotylglycine	34	ND	ND
Catechol	125	150	125
3-Methylcatechol	121	150	125
Benzoate	50	136	5
m-Toluate	31	156	ND
Succinate	ND	ND	107

[a] By Warburg respirometry. Corrected for endogenous rates (10 to 15 μl of O_2 consumed per h). ND, Not determined.

growth of PaM1000 on DL-allylglycine. These same activities were also present in cells of PaM1000 grown on succinate as a carbon source, although the levels of each were somewhat reduced as compared with cells grown on allylglycine. The levels of all *meta* enzymes in PaM1000 grown on succinate were significantly higher than that observed in the parent strain, PaM1, grown similarly (Table 4).

Since growth studies had shown that only the D-isomer of allylglycine supported growth of *P. putida* mt-2, we considered that this might be related to the limited specificity of a D-amino acid dehydrogenase to catalyze the oxidation of D-allylglycine to 2-keto-4-pentenoic acid. As work by Sokatch and co-workers (21-23) had shown that the catabolism of certain D-amino acids by *P. aeruginosa* depended on the ability to synthesize D-specific amino acid dehydrogenases, we tested whether growth on D-allylglycine by *P. putida* mt-2 might also depend on synthesis of a similar activity acting on allylglycine. For this purpose, cell extracts from allylglycine-grown cells of PaM1000 were assayed for the ability to oxidize allylglycine by coupling the

reaction to the reduction of DCPIP as described by Norton et al. (22). Both DL- and D-allylglycine stimulated the rate of DCPIP reduction by crude extracts, but L-allylglycine had no effect (Table 5). Like D-amino acid dehydrogenases described in *Pseudomonas* (21, 35) and *E. coli* (24, 26), we observed that the enzymatic activity toward D-allylglycine could be removed by centrifugation of crude extracts at 100,000 × *g* but was recoverable in the pellet, suggesting that this activity was membrane bound as in other organisms (Table 5). It was further observed that DL-alanine was equal to D-allylglycine in stimulating the rate of DCPIP reduction, whereas DL-*trans*-crotylglycine and DL-α-aminobutyrate were less efficient. The D-amino acid dehydrogenase activity as measured in crude extracts of PaM1000 was shown to be inducible, since no activity was detected in cell extracts prepared from cells grown on succinate alone (Table 5).

Formation and degradation of 2-keto-4-pentenoic acid. Evidence for the formation of 2-keto-4-pentenoic acid as an intermediate in allylglycine degradation by *P. putida* mt-2 was obtained by incubating washed cells of the putative cured strain, PaM3, with allylglycine and demonstrating its accumulation. Cells of PaM3, which had been grown on succinate in the presence of DL-allyglycine, converted D-allylglycine to 2-keto-4-pentenoic acid as evidenced by the accumulation of an unstable chemical species having an absorption maximum (λ_{max}) of 265 nm (Fig. 3; 6, 20). Based on a molar extinction coefficient of 10,000 M^{-1} as described for 2-keto-*cis*-4-hexenoic acid by Coulter and Talalay (6), we estimated that approximately 90% of the D-allylglycine supplied had been converted to 2-keto-4-pentenoate after 60 min of incubation. No spectral evidence for the formation of 2-keto-4-pentenoic acid from L-allylglycine could be obtained. Incubations conducted with DL-allylglycine resulted in the formation of 2-keto-4-pentenoate; however, the yield was estimated at only 50% that observed when D-allylglycine was supplied. It was therefore concluded that only the D-enantiomer of allylglycine served as a substrate for conversion to the keto-enoic acid.

TABLE 4. *Activities of aromatic enzymes in cell extracts*

Enzyme assayed	Sp act (nmol/min per mg) after growth with:		
	PaM1000		PaM1
	DL-Allylglycine	Succinate	Succinate
C230	1,400	200	28
HMSH	740	68	7
HMSD	105	8	0.1
OEH	5,200	540	330
HOA	160	8	4

TABLE 5. *Fractionation of D-amino acid dehydrogenase from P. putida mt-2 (PaM1000)*

Cell fraction used in standard assay[a]	Substrate	Sp act[b]	Relative activity (%)
Succinate + DL-allylglycine			
Crude extract	D-Allylglycine	0.9	22
	L-Allylglycine	<0.1	<2
	DL-Allylglycine	0.44	11
	DL-Alanine	1.2	29
100,000 × *g* supernatant	D-Allylglycine	<0.1	<2
	DL-Alanine	<0.1	<2
100,000 × *g* pellet	D-Allylglycine	4.1	100
	L-Allylglycine	<0.1	<2
	DL-Alanine	4.0	98
	DL-Crotylglycine[c]	1.7	41
	DL-α-Aminobutyrate	2.4	58
Succinate, crude extract	D-Allylglycine	<0.1	<2
	DL-Alanine	<0.1	<2

[a] Assayed by measuring the rate of DCPIP reduction as described in Materials and Methods after growth on the designated carbon sources.

[b] Expressed as nanomoles of DCPIP reduced per minute per milligram of protein.

[c] Represents the *trans* configurational isomer (ICN Pharmaceuticals Inc., Cleveland, Ohio).

When experiments analogous to those described above were carried out with whole cells of PaM1000, the transient appearance of a 265-nm-absorbing species could be detected after 10 min of incubation which then rapidly disappeared within 20 min. It was futher shown that cell extracts of DL-allylglycine-grown cells of PaM1000 rapidly catalyzed the degradation of 2-keto-4-pentenoic acid accumulated by PaM3 (Fig. 4).

Conversion of allylglycine to pyruvate and acetaldehyde. PaM1000 was able to oxidize allylglycine and catalyze the rapid degradation of 2-keto-4-pentenoic acid (Fig. 2; Fig. 4). As cell extracts also contained elevated levels of HOA (Table 4), it seemed appropriate to test whether extracts of PaM1000 might catalyze the complete degradation of allyglycine to the intermediates pyruvate and acetaldehyde. Extracts of PaM1000 were incubated with DL-allylglycine in a 3-ml reaction mixture which contained: 54 mM Tris (pH 7.6; 1.6 ml); 0.67 mM $MgSO_4$ (0.02 ml); 3.3 mM sodium bisulfite (0.4 ml); 16.7 mM DL-allylglycine (0.5 ml); and extract (10 mg of protein; 0.5 ml). After 3 h of incubation at 30°C, the reaction mixtures as well as controls containing either substrate or enzyme alone were acidified with 0.5 ml of 6% $HClO_4$, extracted with ethyl acetate, and analyzed for pyruvate and acetaldehyde. Both compounds were detected

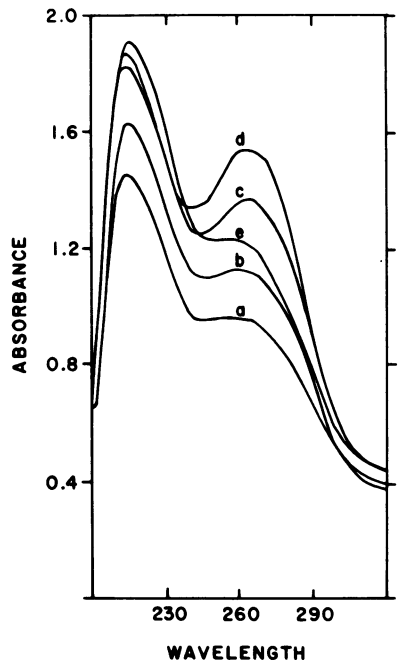

FIG. 3. *Formation of 2-keto-4-pentenoate by whole cells of PaM3 grown on succinate (10 mM) in the presence of* DL-*allylglycine (5 mM). Incubation mixtures contained, in a volume of 30 ml: 23 mM Tris, pH 7.2 (14 ml); 33 mM KCl (10 ml); 0.42 mM* D-*allylglycine (0.5 ml); cell suspension (0.5 ml; 250 mg [wet weight]); and water (5.0 ml). Temperature, 30°C. The reaction was initiated by the addition of cells. At times designated a through e, corresponding to 10, 20, 40, 60, and 120 min, respectively, 0.5-ml samples were withdrawn, acidified with 0.1 ml of 6% $HClO_4$, and centrifuged for 6 min at 10,000 × g. The supernatants were then scanned spectrally after twofold dilutions with water.*

and identified as their 2,4-dinitrophenylhydrazones by thin-layer chromatography (R_f, pyruvate = 0.25, 0.17 [two stereoisomers]; R_f, acetaldehyde = 0.73). No products were detected in control incubations.

In a separate experiment, crude extracts were incubated with DL-allylglycine, and the amounts of pyruvate and acetaldehyde were estimated with lactate and ethanol dehydrogenase, respectively. The reaction conditions were similar to those described above except that the volume was increased to 10 ml and the substrate, DL-allylglycine, supplied at 1 mM. After the addition of substrate, 1-ml samples were withdrawn, heated at 100°C for 3 min, and centrifuged, and the supernatants were analyzed for pyruvate and acetaldehyde. Although the extent of conversion of DL-allylglycine after 3 h was estimated at only about 4%, pyruvate and acetaldehyde were always formed in equimolar proportions. These results are consistent with a pathway for allylglycine degradation involving pyruvate and acetaldehyde as tricarboxylic acid cycle precursors. The relatively low yields of pyruvate and acetaldehyde as measured under these conditions could be due in part to the low rate at which D-amino acid dehydrogenase present in crude extracts attacks DL-allylglycine (Table 5).

DISCUSSION

We have shown that *P. putida* mt-2 is able to degrade D-allylglycine and DL-*cis*-crotylglycine by oxidation to the corresponding keto-enoic acids, the further metabolism of which is effected by TOL plasmid-encoded enzymes (Fig. 1). Cells of *P. putida* mt-2 (PaM1) carrying a TOL plasmid gave rise to mutants able to utilize these amino acids for growth, whereas strains having apparently been cured of their TOL plasmid were unable to do so (Table 2). One such mutant, represented by strain PaM1000, rapidly

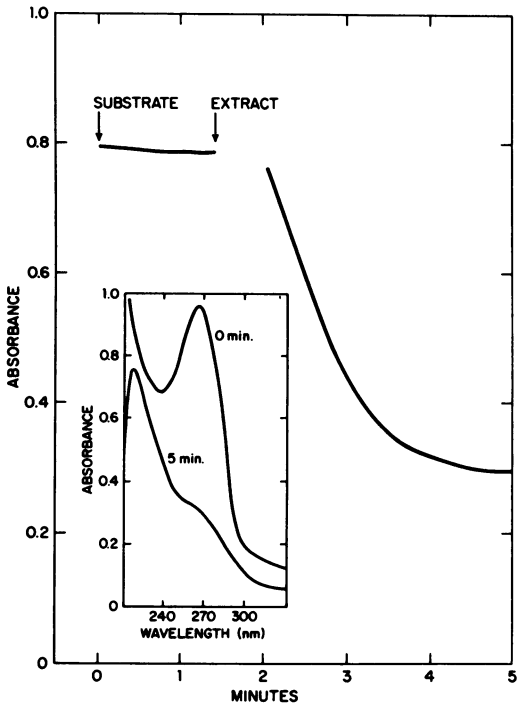

FIG. 4. *Degradation of 2-keto-4-pentenoic acid by cell extracts of PaM1000. The reaction mixture contained, in 3 ml: 38 mM sodium potassium phosphate buffer, pH 7.2 (2.3 ml); 0.33 mM $MnCl_2$ (0.1 ml); approximately 0.1 mM 2-keto-4-pentenoic acid formed biologically as shown in Fig. 3 (0.6 ml); and 5 μl of cell extract (0.1 mg of protein). The reaction mixture was scanned spectrally before the addition of cell extract (0 min) and again at the end of the reaction (5 min).*

and extensively oxidized without lag DL-allylglycine as well as DL-*cis*-crotylglycine, but not L-allylglycine, after growth with DL-allylglycine as the sole carbon source (Fig. 2; Table 3). DL-*trans*-Crotylglycine, although being oxidized at rapid initial rates, underwent only a limited extent of oxidation (Fig. 2). Cell extracts of PaM1000 contained elevated levels of OEH and HOA after growth on DL-allylglycine (Table 4), with the former enzyme showing higher activities toward 2-keto-*cis*-4-hexenoic acid than toward the *trans* isomer. All of these findings are consistent with growth tests which revealed that DL- and D- but not L-allylglycine, and DL-*cis*- but not DL-*trans*-crotylglycine, served as growth substrates for *P. putida* mt-2 carrying a TOL plasmid (Table 2).

2-Keto-4-pentenoate was accumulated in almost equimolar amounts from D-allylglycine when incubated with whole cells of the putative cured strain, PaM3 (Fig. 3). This product was rapidly degraded by cell extracts of PaM1000 (Fig. 4), providing strong evidence that 2-keto-4-pentenoic acid is an intermediate in the metabolism of D-allylglycine. By analogy, 2-keto-*cis*-4-hexenoic acid is proposed as an intermediate in crotylglycine degradation. L-Allylglycine was not oxidized by the cured strain, PaM3. This is consistent with the following observations: (i) the L-isomer of allylglycine could not support growth of strains of PaM1 carrying a TOL plasmid (Table 2), and (ii) cell extracts of PaM1000 catalyzed the oxidation of D- but not L-allylglycine by a D-specific amino acid dehydrogenase (Table 5). These results indicate that utilization of allylglycine and *cis*-crotylglycine by *P. putida* mt-2 is dependent on two separate functions: (i) the ability to elaborate a D-amino acid dehydrogenase responsible for oxidation of the amino acids to the corresponding keto-enoic acids, and (ii) enzymes encoded by TOL plasmids (i.e., OEH and HOA) which facilitate degradation of the keto-enoic acids. The ability of crude cell extracts of PaM1000 to catalyze the complete degradation of allylglycine to pyruvate and acetaldehyde is consistent with such a conclusion.

The observation that D-amino acid dehydrogenase activity elaborated by *P. putida* mt-2 is a particulate enzyme agrees with previous reports wherein the analogous activities for D-alanine and D-valine metabolism in *P. aeruginosa* (21–23) as well as D-alanine dehydrogenase in *E. coli* (24, 26) were shown to be membrane-bound proteins. The finding that this enzyme is synthesized only in response to growth in the presence of allylglycine agrees with other reports documenting the inducible nature of these enzymes. Results which showed that DL-alanine and DL-α-aminobutyrate could serve as substrates for D-amino acid dehydrogenase in addition to D-allylglycine (Table 5) imply either that this activity is nonspecific in its action or that other D-amino acid dehydrogenases such a D-alanine dehydrogenase are synthesized after growth on allylglycine. The former interpretation would be more consistent with the known broad specificity of such enzymes (21, 24). As the putative cured strain, PaM3, retained the ability to oxidize allylglycine to its corresponding keto-enoic acid, it is reasonable to conclude that the D-amino acid dehydrogenase elaborated by *P. putida* mt-2 is a chromosomally encoded enzyme.

The inability of DL-*trans*-crotylglycine to support growth of *P. putida* mt-2 cannot be attributed to an inability on the part of the organism to oxidize this amino acid to 2-keto-*trans*-4-hexenoic acid. This conclusion is based on the following observations: (i) whole cells of PaM1000 were able to oxidize *trans*-crotylglycine after growth on DL-allylglycine, although the extent of oxidation was substantially less than that observed for *cis*-crotylglycine (Fig. 2), and (ii) particulate cell fractions were able to utilize *trans*-crotylglycine as a substrate for D-amino acid dehydrogenase (Table 5). As cell extracts of PaM1000 showed little activity toward 2-keto-*trans*-4-hexenoic acid, the expected product of amino acid oxidation, it seems reasonable to conclude that the inability to utilize this amino acid for growth may be due at least in part to the poor ability to catalyze hydration of the *trans* keto-enoic acid as compared with the *cis* isomer. These results are analogous to those reported by Collinsworth et al. (5), who found that OEH purified from phenol-grown cells of *Pseudomonas* U had little activity toward 2-keto-*trans*-4-hexenoic acid, whereas the *cis* isomer was attacked at rates comparable to those of 2-keto-4-pentenoic acid. One other possible explanation for the lack of growth with *trans*-crotylglycine is that is may not serve as an inducer of D-amino acid dehydrogenase.

The results we report here show a direct relationship between the ability to elaborate enzymes of the catechol *meta* pathway and utilization of allylglycine by *P. putida* mt-2. This association is illustrated by: (i) the absolute dependence on the presence of a TOL plasmid for growth (Table 2); (ii) elevation of the requisite *meta* enzymes (Table 4); and (iii) the ability of cell extracts to catalyze the complete conversion of allylglycine to pyruvate and acetaldehyde. We further observed that other enzymes of the *meta* pathway essential for the catabolism of aromatic compounds, namely, C230, HMSH, and HMSD, were also elevated after growth of PaM1000 on DL-allylglycine (Table 4). Similarly, the toluate oxidase complex as measured in whole cells with

benzoate as a substrate was also elevated under these conditions (Table 3). These results show that growth on DL-allylglycine results not only in an increase in synthesis of OEH and HOA, essential for the catabolism of allylglycine, but also other enzyme activities encoded by the TOL (pWWO) plasmid and associated with catabolism of aromatic substrates. Whether allylglycine can serve as a direct inducer for synthesis of TOL plasmid-encoded enzymes is not yet clear. We also observed that the levels of C230, HMSH, HMSD, OEH, and HOA in PaM1000 grown on succinate were higher than those observed in similarly grown cells of the parent, PaM1 (Table 4). However, the levels of these enzymes as well as that measured for toluate oxidase were not as high as levels seen with allylglycine-grown cells. These results indicate that although the basal levels of the *meta* enzymes in PaM1000 have been increased above those present in the wild-type, PaM1, some other mechanism still exists by which all activities are elevated after growth of PaM1000 on allylglycine. Whether differences in the uninduced levels of the *meta* enzymes in PaM1000 versus PaM1 are somehow related to uninhibited growth of the former strain on allylglycine, whereas the latter must acquire this ability, remains to be resolved. Of further interest is the finding that toluate oxidase, in contrast to C230 and other *meta* enzymes, was not substantially higher in activity in PaM1000 grown on succinate (Tables 3 and 4) than was observed previously for similarly grown cells of PaM1 (17). These findings could imply that the synthesis of toluate oxidase, versus C230 along with other *meta* enzymes, is not controlled coordinately.

The evidence presented here shows that the presence of a TOL plasmid (pWWO or pDK1) in *P. putida* mt-2 confers the ability to utilize allylglycine and *cis*-crotylglycine (Table 2) in addition to toluene, the xylenes, and related hydrocarbons as previously described (17, 18, 36, 38). Why two independently maintained cultures of *P. putida* mt-2 (i.e., PaM1 and PaW1) should behave differently with regard to their ability to readily grow on allylglycine remains to be determined. It is also not clear why other strains of *P. putida* which elaborate the TOL-related degradative pathway appear variable in their ability to utilize allylglycine. However, utilization of the amino acids by exconjugants (e.g., PaM310; Table 2) obtained by mating strain HS1 with the apparent cured strain of mt-2 provides evidence that the TOL (pDK1) plasmid, although not allowing growth in its normal host, does facilitate degradation of these compounds when maintained in the mt-2 host cell background. It may be that *P. putida* mt-2 and *Pseudomonas* Pxy, the latter of which has also been reported to harbor a TOL-type plasmid designated XYL (12) and also gives rise to mutants able to grow on allylglycine and *cis*-crotylglycine (Table 2), are unique in that host cell chromosomally specified D-amino acid dehydrogenases can be utilized to catalyze conversion of the amino acids to the corresponding keto-enoic acids, the further dissimilation of which is made possible by TOL-mediated *meta*-pathway enzymes. Although host cell backgrounds may play an important role in allowing growth on the amino acids, it is possible that other organisms which use the *meta* pathway for the degradation of aromatic molecules may be able to degrade these compounds. Similarly, since 2-keto-4-pentenoate also serves an an intermediate in aromatic catabolic pathways which proceed via 2,3-dihydroxybenzoates as ring fission substrates (28), organisms capable of elaborating this catabolic sequence may also be able to degrade allylglycine.

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