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Aerobic Catabolism of Phenylacetic Acid in *Pseudomonas putida* U: Biochemical Characterization of a Specific Phenylacetic Acid Transport System and Formal Demonstration that Phenylacetyl-Coenzyme A Is a Catabolic Intermediate

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The phenylacetic acid transport system (PATS) of *Pseudomonas putida* U was studied after this bacterium was cultured in a chemically defined medium containing phenylacetic acid (PA) as the sole carbon source. Kinetic measurement was carried out, in vivo, at 30°C in 50 mM phosphate buffer (pH 7.0). Under these conditions, the uptake rate was linear for at least 3 min and the value of K_m was 13 μM. The PATS is an active transport system that is strongly inhibited by 2,4-dinitrophenol, 4-nitrophenol (100%), KCN (97%), 2-nitrophenol (90%), or NaN_3 (80%) added at a 1 mM final concentration (each). Glucose or D-lactate (10 mM each) increases the PATS in starved cells (140%), whereas arsenate (20 mM), NaF, or *N,N'*-dicyclohexylcarbodiimide (1 mM) did not cause any effect. Furthermore, the PATS is insensitive to osmotic shock. These data strongly suggest that the energy for the PATS is derived only from an electron transport system which causes an energy-rich membrane state. The thiol-containing compounds mercaptoethanol, glutathione, and dithiothreitol have no significant effect on the PATS, whereas thiol-modifying reagents such as *N*-ethylmaleimide and iodoacetate strongly inhibit uptake (100 and 93%, respectively). Molecular analogs of PA with a substitution (i) on the ring or (ii) on the acetyl moiety or those containing (iii) a different ring but keeping the acetyl moiety constant inhibit uptake to different extents. None of the compounds tested significantly increase the PA uptake rate except adipic acid, which greatly stimulates it (163%). The PATS is induced by PA and also, gratuitously, by some phenyl derivatives containing an even number of carbon atoms on the aliphatic moiety (4-phenylbutyric, 6-phenylhexanoic, and 8-phenyloctanoic acids). However, similar compounds with an odd number of carbon atoms (benzoic, 3-phenylpropionic, 5-phenylvaleric, 7-phenylheptanoic, and 9-phenylnonanoic acids) as well as many other PA derivatives do not induce the system, suggesting that the true inducer molecule is phenylacetyl-coenzyme A (PA-CoA). Furthermore, after *P. putida* U is cultured in the same medium containing other carbon sources (glucose or octanoic, benzoic, or 4-hydroxyphenylacetic acid) in the place of PA, the PATS and PA-CoA are not detected; neither the PATS nor PA-CoA is found in cases in which mutants (PA^- and PCL^-) lacking the enzyme which catalyzed the initial step of the PA degradation (phenylacetyl-CoA ligase) are used. PA-CoA has been extracted from bacteria and identified as a true PA catabolite by high-performance liquid chromatography and also enzymatically with pure acyl-CoA:6-aminopenicillanic acid acyltransferase from *Penicillium chrysogenum*.

The species of bacteria included in the genus *Pseudomonas* are able to grow in many different chemically defined media containing a wide variety of aromatic molecules as the sole carbon source (7, 9, 14, 26, 36). Recently, we have shown that *Pseudomonas putida* (strain U) can grow very efficiently in a minimal defined medium containing phenylacetic acid (PA) as the sole carbon source (26). Under aerobic conditions, this strain, unlike other *Pseudomonas* strains which also can use PA as an energy source (5, 6, 8, 35), degraded this aromatic compound through a probably new catabolic pathway which involves, as a first step, the activation of PA to its coenzyme A (CoA) derivative (phenylacetyl-CoA [PA-CoA]) (23–26, 35). The existence of a different pathway involved in the specific degradation of PA is supported by two additional pieces of evidence: (i) phenylacetyl-CoA ligase (PCL) was not induced when *P. putida* U was cultured in a defined medium containing either 4-hydroxy-PA or many other phenylacetyl derivatives as the sole carbon source (26), and (ii) PA and 4-hydroxy-PA are catabolized by two unrelated pathways (32). These facts, together with the description of this enzyme (PCL) in other bacteria which also catabolized PA aerobically (35) as well as the identification of PA-CoA inside the bacteria (see below), lend a new perspective to this kind of research.

It therefore seems likely, as has been reported for other microbes (10–12), that the degradation of PA requires the existence of a mechanism (the phenylacetic acid transport system [PATS]) which will permit the uptake of this aromatic compound from the medium. In the present work, we have studied and characterized the PATS in *P. putida* U. Knowledge of this could be important not only to establish the first catabolic step of the route involved in the aerobic catabolism of

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PA but also to understand the molecular mechanism responsible for its in vivo regulation.

MATERIALS AND METHODS

Materials. The following acids were obtained from Aldrich Quimica (Madrid, Spain): 3- and 4-aminophenylacetic acids; 2,5-dihydroxyphenylacetic acid; 2-, 3-, and 4-nitrophenylacetic acids; 2-, 3-, and 4-fluorophenylacetic acids; 2,4-, 2,5-, 2,6-, 3,4-, and 3,6-difluorophenylacetic acids; 2-, 3-, and 4-bromophenylacetic acids; 2-amino-4-thiazoleacetic acid; 2-pyrimidylthioacetic acid; rhodanine-3-acetic acid; 4-imidazolacetic acid; 5-hydantoinacetic acid; 2- and 3-thiopheneacetic acids; thiophenoxyacetic acid; 4-phenoxycarboxyacetic acid; 2-, 3-, and 4-phenylbutyric acids; and 2,4-, 2,6-, and 3,4-dichlorophenylacetic acids. These acids were purchased from Fluka Chemika-Biochemika (Madrid, Spain): 3- and 4-hydroxyphenylacetic acids, 3- and 4-hydroxybenzoic acids, and 4-phenoxybutyric acid. The following acids were supplied by Lancaster MTM Research Chemicals (Strasbourg, France): 2-, 3-, and 4-methylphenylacetic acids; 2-chloro-6-fluorophenylacetic acid; 2-, 3-, and 4-chlorophenylacetic acids; 4-ethoxyphenylacetic acid; 4-hydroxy-4-phenylpropionic acid; phenylglioxylic acid; 2-hydroxy-3-phenylpropionic acid; L-mandelic acid (α-hydroxyphenylacetic acid); 3-hydroxy-3-phenylpropionic acid; 3-phenoxypropionic acid; 2-phenylpropionic acid; 5-phenylvaleric acid; 6-phenylhexanoic acid; 7-phenylheptanoic acid; 8-phenyloctanoic acid; 9-phenylnonanoic acid; 2-, 3-, and 4-pyridylacetic acids; homophthalic acid (2-carboxyphenylacetic acid); homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid); 4-iodophenoxyacetic acid; 3-phenylendiacetic acid; 4-acetylphenoxyacetic acid; and 4-biphenylacetic acid. Benzoic acid and 2-, 3-, and 4-methoxyphenylacetic acids were obtained from Merck (Darmstadt, Germany). Streptomycin sulphate, chloramphenicol, rifampin, kanamycin, 2-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, 3-phenylpropionic acid, PA, phenoxyacetic acid, [1-C^{14}]PA (3.4 mCi/mmol), ATP, CoA, dithiothreitol (DTT), reduced glutathione and oxidized glutathione, reduced 2-mercaptoethanol, *N*-ethylmaleimide, iodoacetate, and PA-CoA lithium salt were from Sigma Chemical Co. (St. Louis, Mo.). [U-^{14}C]leucine (351 mCi/mmol) was purchased from the Radiochemical Centre (Amersham, England), 2-chlorobenzoic acid and chloroacetic acid were supplied by FEROSA (Barcelona, Spain), 4-aminobenzoic acid was obtained from Carlo Erba (Milan, Italy), and DL-phenoxypropionic acid and 2-phenoxybutyric acid were from Janssen (Beerse, Belgium). *Escherichia coli* penicillin acylase was a gift from Antibioticos S.A. (León, Spain). β-Lactamase from *Bacillus cereus* was from Difco (Detroit, Mich.). All other products used were of analytical quality or high-performance liquid chromotography (HPLC) grade.

Strains. *P. putida* (strain U) used in this work was from our collection. The strain was originally obtained from R. A. Cooper (Department of Biochemistry, University of Leicester, Leicester, United Kingdom).

E. coli HB101 containing the plasmid pGS9 (34), which includes the transposon Tn*5*, was kindly supplied by J. L. Ramos (Estación Experimental del Zaidin, Consejo Superior de Investigaciones Científicas, Granada, Spain).

Micrococcus luteus ATCC 9341 was used for the determination of penicillin G (benzylpenicillin) by bioassay (18).

Culture media and growth conditions. *P. putida* was maintained on Trypticase soy agar (Difco), and growth slants (8 h at 30°C) were used to inoculate liquid medium. Each 2,000-ml Erlenmeyer flask containing 250 ml of the required medium (see below) was inoculated with 10 ml of a bacterial suspension (10^{10} bacteria). Incubations were carried out in a rotary shaker (250 rpm) at 30°C for the time required in each set of experiments. The medium (MM) used for growth of *P. putida* was a chemically defined one (26). Its composition was the following (in grams per liter): KPO_4H_2, 13.6; $(NH_4)_2SO_4$, 2.0; $MgSO_4 \cdot 7H_2O$, 0.25; $FeSO_4 \cdot 7H_2O$, 0.0005; and PA, 0.68. When necessary, (i) the medium was enriched with an additional carbon source, (ii) the carbon source was replaced by a different one, or (iii) the concentration of PA was changed. For solid media, 25 g of agar (Difco) $liter^{-1}$ was added. Each plate (9 cm in diameter) contained 28 ml of the required medium.

PA uptake in *P. putida* U. Cells grown in the above-reported medium were harvested at different times, washed twice with sterile distilled water, and resuspended in 50 mM phosphate buffer (pH 7.0). Aliquots of 1.4 ml (1.4×10^9 bacteria) were placed in 25-ml Erlenmeyer flasks and preincubated at 30°C for 3 min in a thermostatically controlled bath at 160 strokes per min before PA (21.4 μM containing 9.9 μM labelled PA) was added). Incubations were carried out for different periods of time (usually 1 min), halted by the addition of 10 vol of water, rapidly filtered through Millipore filters (pore size, 0.45 μm), and washed with 2×10 ml of sterile distilled water. The filters were dissolved in 10 ml of scintillation fluid and counted as described (11, 29, 33). Effectors were added to 1 mM unless indicated otherwise. In these cases, the effector tested was added 2 min before PA was added.

Determinations of the PATS half-life. Cells were grown in MM as described above, and at 10 h, or at the required time, protein synthesis (PTS) was stopped by adding 75 μg of chloramphenicol ml^{-1}. From this time to 15 h, the PATS was measured at 30-min intervals.

Cell-shocking procedure. Osmotic shock was carried out by the procedure reported by Neu and Heppel (31) and slightly modified by Rodríguez-Aparicio et al. (33).

PATS induction experiments. *P. putida* U was grown in MM in which the PA had been replaced by an alternative growth substrate (5 mM): octanoic acid, succinic acid, benzoic acid, 4-hydroxy-PA, one of several phenyl derivatives, glycerol, acetate, or glucose. When *P. putida* was not able to catabolize a certain compound, the compound inducer capacity was studied by growing the bacteria in MM plus glucose (5 mM) in the place of PA. Once the glucose had been exhausted, the molecule to be tested (1 mM) was added to the culture. The appearance of PATS was monitored at intervals.

Enzymatic assays. Cell extracts of *P. putida* were obtained as reported elsewhere (26). PCL was assayed by measuring the rate of phenylacetylhydroxamate formation in the presence of ATP, CoA, PA, and neutral hydroxylamine (26). In some cases (formation of PA-CoA or other phenyl thioesters), the enzymatic assays were carried out in the absence of hydroxylamine and the reaction products were analyzed by HPLC (see below). In such cases, reactions were stopped by adding methanol (50%, vol/vol). To analyze the intracellular levels of PA and PA-CoA, cell extracts were centrifuged ($12,000 \times g$ for 10 min), precipitated with ammonium sulfate (65% saturation) to eliminate protein, and ultracentrifuged ($45,000 \times g$ for 60 min). The supernatant was lyophilized and analyzed by HPLC.

Analytical determinations. Residual D-glucose was measured by the glucose oxidase enzymatic test (17). The utilization of PA or 4-hydroxy-PA by *P. putida* was monitored by measurement by HPLC of the disappearance of these compounds from the culture broths (see below). In some experiments, the presence of [^{14}C]PA or the formation of labelled PA derivatives inside the cells was analyzed by the same procedure.

TABLE 1. Effects of several uncouplers, metabolic inhibitors, and thiol-containing and thiol-modifying reagents on the PATS in *P. putida*[a]

Effector[b]	PA uptake (%)[c]
Control	100
2-Nitrophenol (1 mM)	10
4-Nitrophenol (1 mM)	ND
2,4-Nitrophenol (1 mM)	ND
Potassium cyanide (KCN) (1 mM)	3
Sodium azide (NaN_3) (1 mM)	20
N-Ethylmaleimide (1 mM)	ND
Iodoacetate (1 mM)	7
Potassium arsenate (20 mM)	100

[a] The data reported in this table are the averages of three different assays.
[b] Reduced glutathione, oxidized glutathione, reduced 2-mercaptoethanol, and DTT did not cause any effect.
[c] A labelled PA uptake of 100% corresponds to 2,500 pmol/min. The lowest limit of detection was 75 pmol/min (blank). ND, no detection.

TABLE 2. PA uptake by starved cells of *P. putida* when incubated with D-glucose or D-lactate in the presence of different energy inhibitors[a]

Addition[b]	PA uptake (pmol/min)
None	2,500
Glucose	3,776
Glucose and NaF	3,632
Glucose and KCN	196
Glucose and *N,N'*-dicyclohexylcarbodiimide	3,158
Glucose and 4-nitrophenol	188
D-Lactate	3,575
D-Lactate and NaF	3,326
D-Lactate and KCN	173
D-Lactate and *N,N'*-dicyclohexylcarbodiimide	2,558
D-Lactate and 4-nitrophenol	83

[a] The data reported in this table are the averages of three different assays.
[b] Glucose (10 mM) and D-lactate (10 mM) were added to the cultures and incubated (10 min) before the uptake rates were measured. The different inhibitors (NaF, KCN, *N,N'*-dicyclohexylcarbodiimide, and 4-nitrophenol at 1 mM each) were added to the uptake mixture 3 min before [^{14}C]PA.

Determination of PTS. PTS in *P. putida* U was monitored by measuring the incorporation of [U-^{14}C]leucine into trichloroacetic acid-insoluble materials, as has been reported for other biological systems (21, 22). PTS was stopped by adding one of the following antibiotics to culture cells grown in MM: kanamycin, chloramphenicol, or streptomycin (75 μg ml^{-1} each). Although in some cases the three antibiotics were employed, chloramphenicol was routinely used.

Isolation of mutants unable to catabolize PA. Mutations were produced by insertion of the transposon Tn*5* (2, 3) in some of the genes involved in the degradation of PA, and the insertions were done by previously reported procedures (13, 15, 32). The induction of PATS or PCL in these mutants was studied by growing them in MM containing 5 mM 4-hydroxy-PA (to support bacterial growth) and 2.5 mM PA (as an inducer of PATS and PCL) as carbon sources.

Isolation of PA-CoA from bacteria. Bacteria grown as above were harvested at the logarithmic phase of growth by centrifugation at 10,000 × *g* for 5 min at 4°C. Cells from 2.5 liters of bacterial cultures (4×10^{12} bacteria) were mixed and extracted by gentle stirring with 50 ml of ethanol. Bacterial cells were removed by centrifugation (10,000 × *g*, 10 min), and the supernatant was kept under refrigeration (−20°C). Aliquots of 50 μl were analyzed by HPLC (see below).

The supernatants from 10 different experiments (25 liters of bacterial cultures [4×10^{13} bacteria]) were mixed, diluted with a similar volume of water, frozen at −40°C, and lyophilized. The solid obtained was resuspended in 30 ml of distilled water and centrifuged at 35,000 × *g* for 10 min at 4°C to eliminate insoluble materials, and the supernatant (27 ml) was mixed with a similar volume of methanol. The mixture was kept on ice for 30 min and centrifuged again (35,000 × *g*, 10 min, 4°C). The precipitate was discarded, and the supernatant was used to measure PA-CoA by HPLC. This fraction was lyophilized and stored under refrigeration until required. By this procedure, 1.1 mg of PA-CoA was obtained.

Acyl-CoA:6-aminopenicillanic acid (6-APA) acyltransferase (AT) assay. AT was purified from the fungus *Penicillium chrysogenum* Wis 54-1255 as previously reported (1). In a total volume of 100 μl, the reaction mixture contained the following: 50 mM Tris-HCl buffer (pH 8.2), 25 μl; 10 mM PA-CoA (or the sample to be analyzed), 10 μl; 0.3 mM 6-APA, 10 μl; 30 mM DTT, 5 μl; and enzyme (AT), 50 μl (0.05 mg of protein). Incubations were carried out at 20°C for 30 min and stopped by adding 100 μl of methanol. When required, *E. coli* penicillin acylase (4 IU) or β-lactamase from *B. cereus* (10 μl) was added to the reaction mixture to determine the sensitivity of the antibiotic synthesized to these two enzymes. The quantity of benzylpenicillin produced was evaluated by bioassay against *M. luteus* ATCC 9341, as previously reported (18).

HPLC equipment and chromatography procedure. To measure the rate of PA utilization and to establish the substrate specificity of PCL (formation of CoA derivatives in the presence of different analogs of PA able to induce PATS and PCL), samples of culture broths or of the PCL reaction mixtures were taken at different times, centrifuged, and filtered through a Millipore filter (pore size, 0.45 μm). Aliquots of 50 μl were analyzed with an HPLC apparatus (SP 8800; Spectra Physics) equipped with a variable wavelength UV/VIS detector (SP 8450), a computing integrator (SP 4290), and a microparticulate (particle size, 10 μm; pore size, 100 Å [10 nm]) reverse-phase column (Nucleosil C-18, 4.6 [inner diameter] by 250 mm; Phenomenex Laboratories). The mobile phases used for the separation of PA, different phenyl derivatives, and their CoA thioesters were as follows. (i) The mobile phase for PA, benzoic acid, PA-CoA, and benzoyl-CoA was 0.2 M KH_2PO_4 (pH 4.5)–2-propanol (90:10, vol/vol); the retention times (Rt) for each compound were 14, 19, 22, and 25 min, respectively. (ii) For 3-phenylpropionic, 4-phenylbutyric, and 5-phenylvaleric acids, the mobile phase was 0.2 M KH_2PO_4 (pH 4.5)–CH_3CN (70:30, vol/vol); the Rt were 12, 19, and 34 min, respectively. (iii) The mobile phase for 6-phenylhexanoic, 7-phenylheptanoic, and 8-phenyloctanoic acids was 0.2 M KH_2PO_4 (pH 4.5)–CH_3CN (60:40, vol/vol); the Rt were 22, 35, and 59 min, respectively. (iv) For 9-phenylnonanoic acid, the mobile phase was 0.2 M KH_2PO_4 (pH 4.5)–CH_3CN (50:50, vol/vol); the Rt was 32 min.

In all cases, the flow rate was 1 ml min^{-1}. For the analysis of 4-hydroxy-PA, the mobile phase was 0.05 M KH_2PO_4 (pH 4.0)–CH_3CN (99:1, vol/vol). The flow rate was set at 2.5 ml/min, and the eluate was monitored, as in the preceding cases, at 254 nm.

RESULTS AND DISCUSSION

Time course of the appearance and characteristics of the PATS. The PATS was measured in *P. putida* U grown for 10 h in the above-reported chemically defined medium (MM).

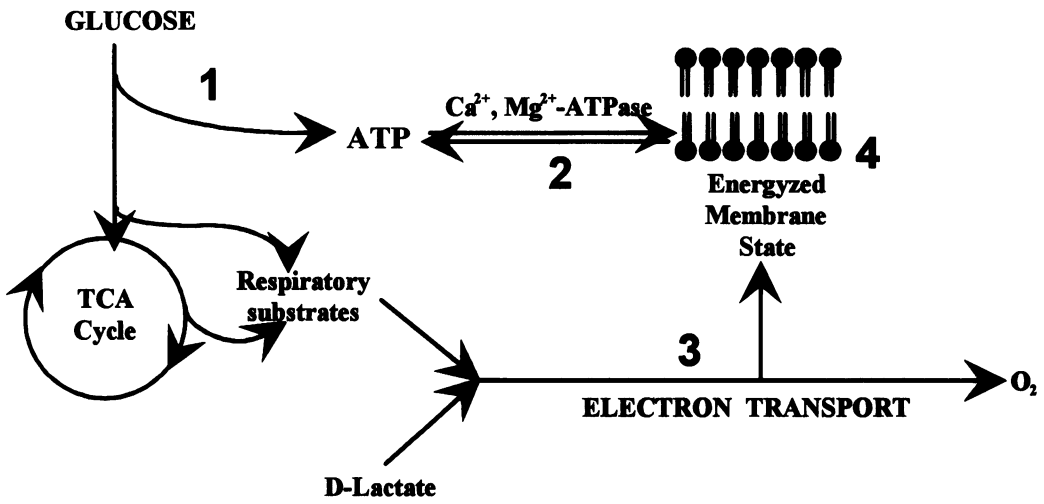

FIG. 1. Energy flow for the active transport of PA. The numbers indicate the levels at which different molecules inhibit the obtaining of energy. 1, Sodium fluoride and potassium arsenate. 2, *N,N*-dicyclohexylcarbodiimide. 3, Potassium cyanide. 4, 2-Nitrophenol, 4-nitrophenol, 2,4-dinitrophenol, and sodium azide. TCA, tricarboxylic acid cycle.

Determination of its optimal physicochemical parameters showed that the maximal uptake rate was reached at 30°C in 50 mM phosphate buffer (pH 7.0), and under these conditions, PA incorporation was linear for at least 3 min; the calculated K_m was 13 μM. The half-life of PATS (see Materials and Methods) was 2 h, suggesting that (i) no substantial changes occur in the PATS during this period and (ii) the PATS has a fast rate of turnover compared with that of other similar or unrelated transport systems (11, 33). PA uptake was inhibited by KCN, 2,4-dinitrophenol, 4-nitrophenol, and 2-nitrophenol, whereas arsenate, even at 20 mM, did not cause any significant effect (Table 1). These results, like those reported by us for the PATS in *P. chrysogenum* Wis 54-1255 (11) or by Hunter and Segel studying the uptake of basic amino acids in this fungus (16), suggest that the PATS assayed in *P. putida* is an energy-dependent system. The lack of inhibition caused by arsenate and NaF (Tables 1 and 2) further suggests that endogenous ATP is not the energy source for this active transport system. Moreover, the sensitivity to cyanide and several uncouplers suggests that the PATS is driven directly by an energy-rich membrane state that is generated by electron transport. The ability of glucose or D-lactate to provide energy for active transport in starved *P. putida* cells, blocked at specific sites of energy metabolism, was studied (Fig. 1 and Table 2). Glucose can give rise to an energy-rich membrane state by two different pathways: through hydrolysis of glycolytic ATP by ATPase in the presence or absence of respiration or through oxidations of the respiratory chain in the absence of ATPase. Alternatively, D-lactate is oxidized directly by a membrane-bound dehydrogenase coupled to the cytochrome chain and can provide energy only in the presence of electron transport (4). The data included in Table 2 indicate that the energy for PA transport can only be derived from the electron transport system and not from glycolysis. The model proposed here is also coherent with the insensitivity of the PATS to osmotic shock (data not shown).

Another argument for the active uptake of PA emerges from the results obtained when the efflux of PA was measured in the presence of the protonophore 2,4-dinitrophenol. Cells were incubated under standard conditions, and 2,4-nitrophenol was added after 3 min of uptake. No efflux of labelled PA was detected even after 15 min of incubation, suggesting that a

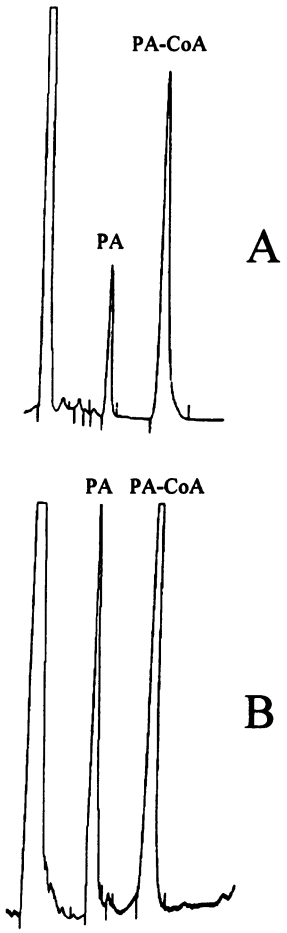

FIG. 2. HPLC chromatograms of (A) PA and PA-CoA and (B) the products obtained from *P. putida* U when bacteria grown in a chemically defined medium containing PA as the sole carbon source were extracted with ethanol (see Materials and Methods).

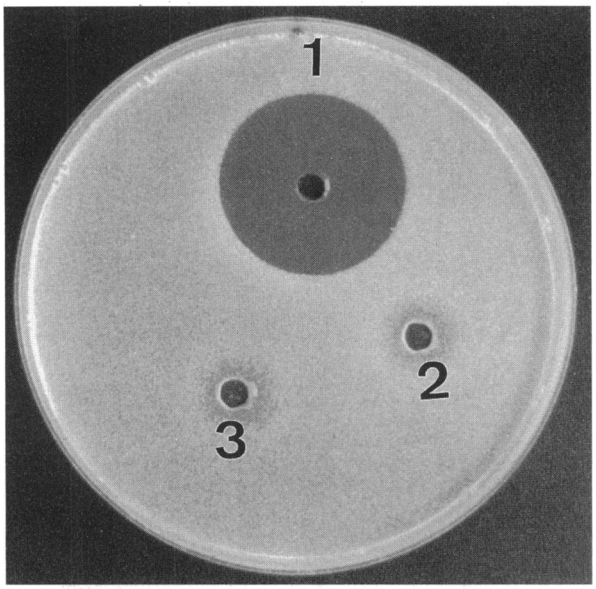

FIG. 3. Bioassay against *M. luteus* ATCC 9341 of (1) the product generated after incubating AT from *P. chrysogenum* Wis 54-1255 with 6-APA, DTT, and the PA-CoA obtained by ethanolic extraction. Numbers 2 and 3 correspond to identical reactions in which β-lactamase from *B. cereus* or *E. coli* penicillin acylase were added, respectively. The inhibition zone is equivalent to 0.5 IU of benzylpenicillin (penicillin G) ml^{-1}. When 6-APA, PA-CoA, or AT were omitted, antibiotic was not formed.

passive PA system does not exist in *P. putida* U (results not shown). HPLC study of the labelled material accumulated inside the cells after 3 min of uptake revealed that 80% of the radioactive material was found as PA, whereas only 20% was identified as a labelled peak with the same retention time as PA-CoA (see below). These data indicate that most of the PA is taken up by *P. putida* without modification and that later it is transformed to PA-CoA to be catabolized. The existence of PA-CoA as a true catabolite intermediate in the aerobic degradation of PA was determined as indicated in Materials and Methods. Figure 2 shows that when *P. putida* U was cultured in the above chemically defined medium and extracted with ethanol, two different peaks with identical retention times to that of true PA (14 min) and PA-CoA (22 min) appeared.

Although the characterization of PA-CoA by HPLC could be considered as formal evidence and consistent proof of its in vivo synthesis by bacteria grown aerobically in a medium containing PA, it does not escape us that it is also possible that a different compound with the same retention time as that of PA-CoA could be extracted. Thus, the peak showing a retention time of 22 min would not be true PA-CoA but rather another compound. To clarify this point, we used a very specific method of analysis to evaluate the presence of PA-CoA. The procedure uses the lyophilized product isolated after several ethanolic extractions (see Materials and Methods) as a substrate for the enzyme AT from *P. chrysogenum* Wis 54-1255. The enzyme, which has been characterized and purified by us (1, 19, 20), catalyzes the following reaction: PA-CoA + 6-APA $\xrightarrow{DTT}$ benzylpenicillin (penicillin G) + CoA. Incubation of AT with the sample to be analyzed, in the presence of 6-APA and DTT, generated a reaction product with antibacterial activity against *M. luteus*, and like penicillin G, the product was sensitive to *B. cereus* β-lactamase and also to *E. coli* penicillin acylase (Fig. 3). These results unequivocally demonstrate that the compound obtained through ethanolic extraction of *P. putida* cells is true PA-CoA. Other structural molecules similar to PA-CoA either have different retention times in HPLC chromatograms or are not substrates for AT.

TABLE 3. Presence of PCL, PATS, and PA-CoA in *P. putida* grown in a chemically defined medium containing different carbon sources[a]

Carbon source (5 mM)	PCL activity[b]	PATS[c]	PA-CoA[d]
PA	43	2,325	110
Benzoic acid	ND[e]	ND	ND
4-Hydroxy-PA	ND	ND	ND
Octanoic acid	ND	ND	ND
Glucose	ND	ND	ND

[a] The data reported in this table are the averages of three different assays.
[b] PCL activity is given as nanomoles of phenylhydroxamate per minute per milligram of protein (26).
[c] PATS is given as picomoles of PA per minute.
[d] PA-CoA is given as micrograms obtained after cells harvested from 10 Erlenmeyer flasks were extracted with ethanol (A_{540}) values ranging between the equivalents of 1.6×10^{13} and 4×10^{13} bacteria).
[e] ND, no detection.

Furthermore, when bacteria were cultured in the same medium in which PA had been replaced by other molecules (4-hydroxy-PA, benzoic or octanoic acids, or glucose), PA-CoA was not found (Table 3). Other results offering further insight into our hypothesis were obtained when mutants handicapped in the catabolism of PA (PA^-) were used. All these Tn*5*-containing mutants were able to grow in the chemically defined medium when PA was replaced by 4-hydroxy-PA, suggesting that neither aromatic compound is catabolized by either the same or by a partially common pathway (32). These mutants have been classified in two different groups: PA strains containing a functional PCL (PA_{1-3}, PA_5, and PA_8 [chemotype PA^-, subclass PCL^+]) and those without this enzymatic activity (PA_4, PA_6, PA_7, PA_9, and PA_{10}). When these mutants were cultured until the mid-logarithmic phase of growth ($A_{540} = 0.9$, 0.9×10^9 bacteria per ml) in minimal medium containing 4-hydroxy-PA (for supporting bacterial growth) and PA (as the source of catabolic intermediates) and extracted with ethanol, PA-CoA was found in all the mutants classified as PCL^+,

TABLE 4. Presence of PA-CoA in the ethanolic extractions of either *P. putida* U (wild type) or PA mutants (Tn*5* mutants) grown in a chemically defined medium[a]

Strain	Chemotype	PCL activity[b]	PA-CoA[c]
P. putida U (wild type)	PA^+ PCL^+	22	35
Mutants $PA_{4,6,7,9,10}$	PA^- PCL^-	ND[d]	ND
Mutant PA_1	PA^- PCL^+	17	15
Mutant PA_2	PA^- PCL^+	15	47
Mutant PA_3	PA^- PCL^+	19	18
Mutant PA_5	PA^- PCL^+	15	60
Mutant PA_8	PA^- PCL^+	38	47

[a] The data reported in this table are the averages of three different assays. The medium contained 4-hydroxy-PA (0.63 g $liter^{-1}$) and PA (0.34 g $liter^{-1}$) as the carbon sources.
[b] PCL activity is given as nanomoles of phenylhydroxamate per minute per milligram of protein.
[c] PA-CoA is given as micrograms obtained after cells harvested from 10 Erlenmeyer flasks were extracted.
[d] ND, no detection.

TABLE 5. Effects of different PA derivatives or analogs on the PATS of *P. putida*[a]

Effector	PA uptake (%) at final concn of[b]:		
	1 mM	100 μM	10 μM
2-Fluorophenylacetic acid	ND	4	34
2,4-Fluorophenylacetic acid	ND	9	62
2-Bromophenylacetic acid	10	80	
3-Bromophenylacetic acid	10	84	
4-Bromophenylacetic acid	8	71	
2-Phenylpropionic acid	9	43	
2-Pyridylacetic acid	8	13	26
2-Thiopheneacetic acid	ND	19	81
Thiophenoxyacetic acid	ND	54	
Rhodanine-3-acetic acid	130	114	
Adipic acid	163	130	100

[a] The data reported in this table are the averages of three different assays.
[b] All the effectors were tested at 1 mM, and in some cases, 100 and 10 μM final concentrations were used. The lowest limit of detection was 75 pmol/min (blank). ND, no detection.

whereas it was absent in the chromatograms corresponding to mutants (PA^-) showing a PCL^- chemotype (Table 4). In summary, these data indicate that PA-CoA is only synthesized when PA is being catabolized.

Effect of different phenyl derivatives and other molecules on PA uptake. The stimulation or inhibition of the PATS by several effectors was examined by adding these compounds (1 mM each) to the uptake mixture. All the molecules that had no effect at such concentrations were discarded as inhibitors, whereas those which had some negative effect were assayed at a lower concentration. All the compounds tested were classified in four different groups according to their chemical structure: (i) analogs of PA with substitution on the ring (compounds in which one or more hydrogen atoms of the aromatic ring had been replaced by hydroxy, methyl, methoxy, amino, or nitro groups or halogen atoms); (ii) compounds in which the acetyl group of PA had been shortened (benzoic acid and derivatives) or lengthened (3-phenylpropionic, 4-phenylbutyric, and other acids); (iii) molecules containing the same ring and acetyl moiety but with substitution on the latter; and (iv) molecules in which the ring of PA had been replaced by others but which kept the acetyl moiety constant. The results obtained with all of them are summarized in Table 5. Only the compounds which caused inhibitions higher than 90% on the uptake rate, at a 1 mM final concentration, are included in Table 5. Among the compounds included in the first group (i), maximal inhibition was caused by halogen derivatives of PA, especially when an atom of F was the substituent. This effect could be explained by the similarities of PA with these compounds, e.g., the size of the substituent is not large enough to hinder its location in the active site of the transporter. These results are quite similar to those reported after study of the substrate specificity of the enzyme PCL; however, in this case, Br derivatives of PA were not used as substrates and this enzyme was strongly inhibited by chloro-PA derivatives (24). When the PATS was incubated with Br and Cl derivatives (at 1 mM), the uptake of PA was partially inhibited, whereas at lower concentrations these compounds were ineffective.

The compounds included in the second and third groups (ii and iii) caused lower degrees of inhibition, with 2-phenylpropionic acid being the only compound which caused an effect greater than 90%. The structural similarity of this compound (α-methylphenylacetic acid) and PA could be the reason for such inhibition. However, either substitution at the same

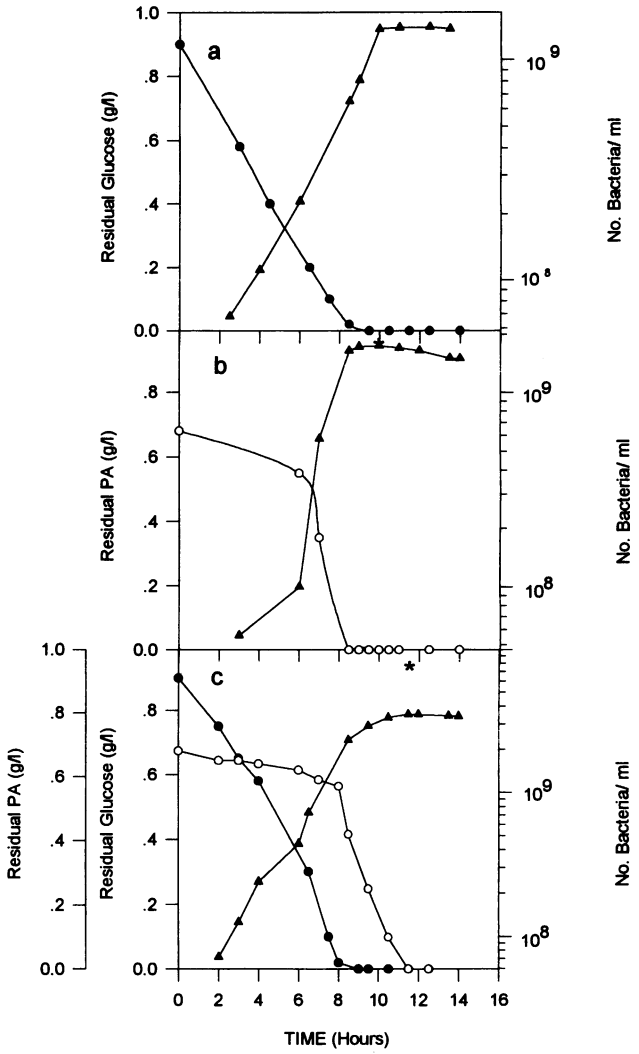

FIG. 4. Bacterial growth (▲) and residual glucose (●) or PA (○) when *P. putida* was grown in MM containing (a) 5 mM glucose, (b) 5 mM PA, or (c) 5 mM glucose and 5 mM PA as carbon sources. *, maximal uptake rate (2,500 pmol/min).

position (α-carbon atom of PA) of a hydrogen atom by an ethyl group (2-phenylbutyric acid or α-ethylphenylacetic acid), which represents an increase in molecular size and volume with respect to α-methylphenylacetic acid, or the presence of a hydrophilic group (hydroxy group in mandelic acid) caused a smaller effect on the PATS (50%).

Finally, the maximal inhibitory effects elicited by the compounds containing a different ring (iv) were induced by derivatives of thiophene and by 2-pyridylacetic acid, whereas other close molecules such as α-amino-2-thiopheneacetic acid or pyridyl derivatives with the acetyl moiety in a different position (3 or 4) caused considerably lower degrees of inhibition (57 and 30%, respectively).

None of the molecules included in these groups stimulated PA uptake significantly (more than 10%) except for rhodanine-3-acetic acid, which, for unknown reasons, increased it to a higher extent (Table 5).

Effect of thiol-containing and thiol-modifying reagents on the PATS. The effects of exogenously added monothiols (reduced glutathione and reduced 2-mercaptoethanol) and dithi-

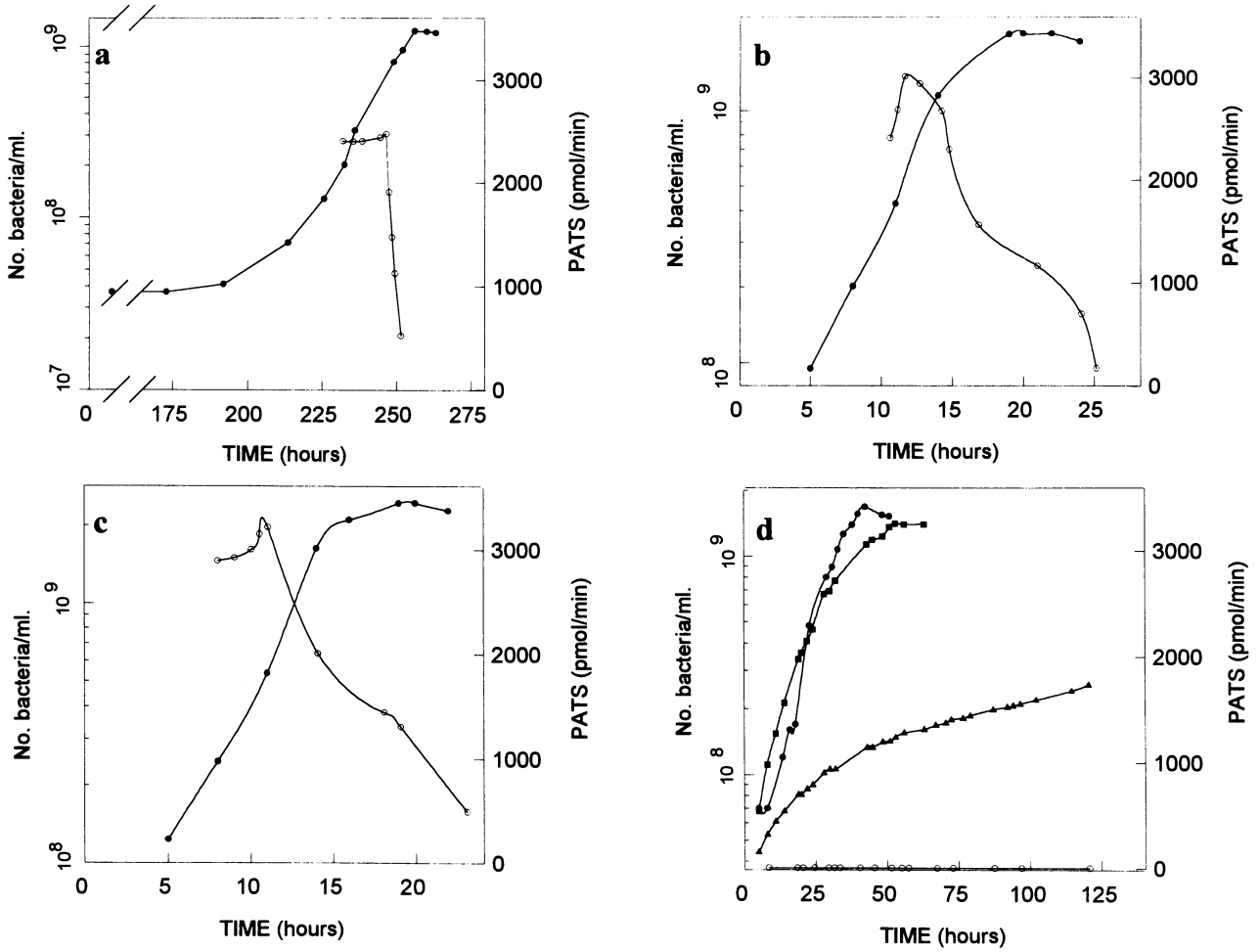

FIG. 5. PA uptake rates (○) and bacterial growth when *P. putida* U was grown in MM containing different phenyl derivatives (5 mM) as carbon sources. (a) 4-Phenylbutyric acid (●). (b) 6-Phenylhexanoic acid (●). (c) 8-Phenyloctanoic acid (●). (d) 5-Phenylvaleric acid (▲), 7-phenylheptanoic acid (■), and 9-phenylnonanoic acid (●). *P. putida* did not grow in MM containing 3-phenylpropionic acid as the sole carbon source, even after 12 days of incubation.

ols (oxidized glutathione and DTT) on the PATS were studied by adding these molecules to the reaction mixture (final concentration, 1 mM). Cells were preincubated in the presence of the effector for 2 min, after which [^{14}C]PA was added (see Materials and Methods). None of these compounds had any significant effect on the rate of transport, suggesting that an extracellular environment containing reduced thiols might not be necessary for cellular incorporation of PA and indicating that maintenance of membrane-associated disulfide groups (which must be present in an oxidized state) is not necessary for the activity of the PATS (30). However, the thiol-modifying reagents *N*-ethylmaleimide and iodoacetate strongly inhibited the uptake of PA (by more than 90%) (Table 1). This suggests that reduced thiol groups are essential for the catalytic function of the PATS. Data similar to these have been reported for the uptake of PA in *P. chrysogenum* Wis 54-1255 (11) and in other transport systems (16, 29, 33).

Effect of fatty acids on the PATS. The PATS in *P. chrysogenum* was strongly inhibited by fatty acids, with this effect being maximal when the carbon length of such compounds increased and which is probably due to the irreversible damage caused by these detergents on the membranes of this fungus (11). However, in *P. putida*, unlike *P. chrysogenum*, the PATS was inhibited to a very low extent. The effect observed was maximal (between 50 to 70% of inhibition) when short fatty acids were added to the uptake mixture, whereas those with a carbon chain longer than C_6 caused only a slight degree of inhibition (lower than 30%). This ineffectiveness indicates that the membrane permeability of *P. putida* is quite different from that of other microorganisms (11, 16, 29, 31, 33) and could be one of the reasons for (i) the natural resistance of this bacterium to these kinds of molecule as well as (ii) its ability to grow efficiently in minimal media containing these aliphatic acids as the sole carbon source (12). This property gives *P. putida* a considerable ecological advantage since it contributes to the possibility of colonizing habitats which, owing to the presence of detergents, are toxic for many other microbes.

It is interesting to note the effect that single structural variations in the molecules of some fatty acids (hexanoic, heptanoic, and octanoic acids) can cause on the PATS. Thus, replacement of the methyl group by a carboxy group (adipic, pimelic, and suberic acids) means that whereas two of the fatty acids (pimelic and suberic acids) only cause a slight stimulatory effect (15%), the other, adipic acid, strongly increases the rate

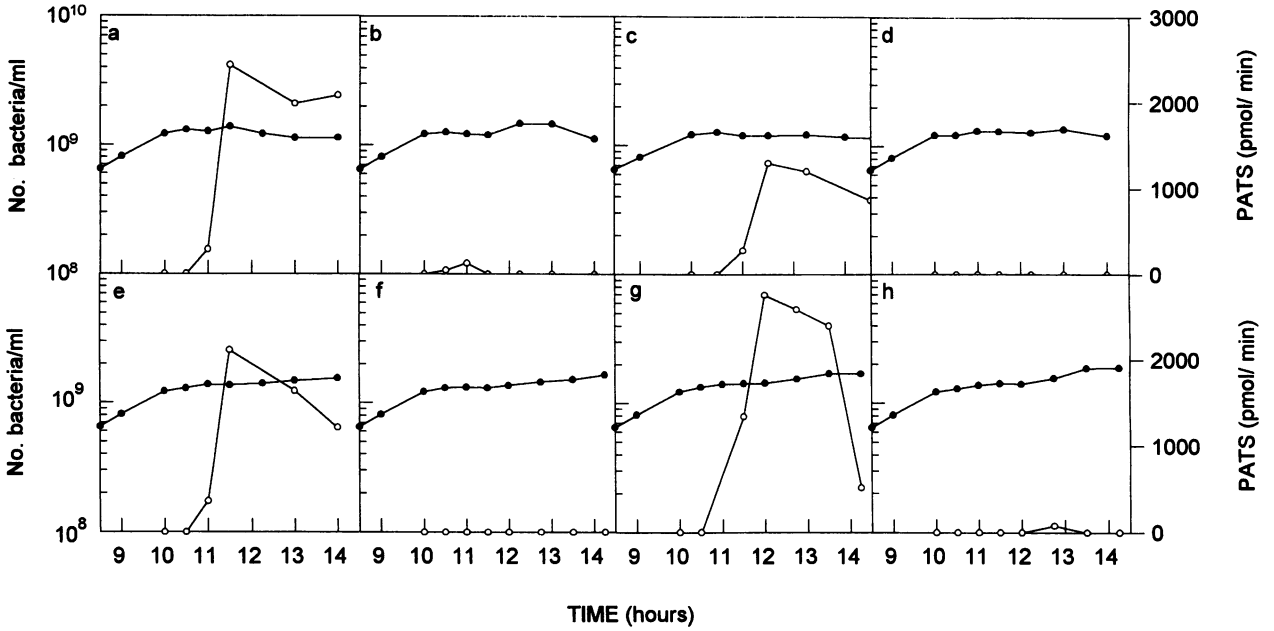

FIG. 6. Study of PATS induction in *P. putida* grown in MM with 5 mM glucose for 10 h. At the 10-h mark, different phenyl derivatives (1 mM each) were added. (a) PA, (b) 3-phenylpropionic acid, (c) 4-phenylbutyric acid, (d) 5-phenylvaleric acid, (e) 6-phenylhexanoic acid, (f) 7-phenylheptanoic acid, (g) phenyloctanoic acid, and (h) 9-phenylnonanoic acid. ●, bacterial growth; ○, PA uptake. α-Methyl-PA (2-phenylpropionic acid) and α-ethyl-PA (2-phenylbutyric acid) as well as those phenyl derivatives containing an odd number of carbon atoms in the acyl chain (panels b, d, f, and h) were unable to induce the PATS.

of uptake (163%). This effect seems to be quite specific for the PATS since adipic acid did not stimulate the activity of PCL in vitro.

Induction of the PATS. The induction of the PATS was studied by growing *P. putida* in minimal medium containing different compounds (glucose, octanoic acid, glycerol, acetic acid, succinic acid, and aromatic acids) as the sole carbon sources. The PATS only appeared when PA or some phenyl derivatives (see below) were the carbon sources, whereas it did not with glucose, octanoic acid, acetic acid, glycerol, succinic acid, 4-hydroxy-PA, and many other energy sources. Moreover, when *P. putida* was cultured in MM containing PA (5 mM) and glucose (5 mM), PA did not start to be catabolized until the glucose had been exhausted (Fig. 4b and c), and therefore induction of the PATS was delayed (data not shown). When different aromatics were tested as carbon sources, we observed that *P. putida* can efficiently use a small number of them (benzoic acid and 6-phenylhexanoic, 7-phenylheptanoic, 8-phenyloctanoic, and 9-phenylnonanoic acids), others were only poorly used (4-phenylbutyric acid and 5-phenylvaleric acid), and 3-phenylpropionic acid was not metabolized (Fig. 5). Thus, to study their ability to induce the PATS, bacteria were grown in MM (without PA) containing 5 mM glucose as the sole carbon source, and at 10 h, when the glucose was exhausted, the molecule to be tested was added (1 mM) (see Materials and Methods). The PATS was later assayed at different intervals. Figure 6 shows that only those phenyl derivatives containing an even number of carbon atoms in the acyl chain (4-phenylbutyric, 6-phenylhexanoic, and 8-phenyloctanoic acids) were able to induce the PATS. Furthermore, the fact that the PATS, which does not seem to be a transport system involved in the uptake of phenyl derivatives containing more than two carbon atoms in the acyl chain linked to the aromatic moiety, could be assayed along the growth curve when *P. putida* was cultured in MM containing such compounds as carbon sources reinforces the idea that it is gratuitously induced by these molecules. Moreover, these compounds also induce (as in the case of PA) the synthesis of the enzyme PCL, suggesting that PATS and PCL genes are coordinately transcribed. The fact that none of these compounds was activated by PCL to the corresponding CoA thioesters (undetectable by HPLC) and that none of them inhibited the uptake of PA at 100 μM (a concentration fivefold higher than that of PA) suggests that these molecules gratuitously induce at least the first two steps of the degradative pathway of PA (data not shown). It is interesting to note that benzoic acid and phenyl derivatives containing an odd number of carbon atoms (3-phenylpropionic, 5-phenylvaleric, 7-phenylheptanoic, and 9-phenylnonanoic acids) were not able to induce the PATS. Assuming that the inducer molecule is able to exert such a function on the basis of its chemical structure (presence of a benzene ring without substitution and an aliphatic chain terminating in a carboxy group), one would expect that molecules containing an even or odd number of carbon atoms on the acyl moiety linked to the ring or, at least, those containing an aliphatic length ranging between certain limits (C_2 to C_8) would all be able to induce the PATS. Results similar to these have been reported for other enzymatic systems that use analogous molecules as substrate (1, 20, 27, 28). However, the fact that only compounds containing an even number of carbon atoms were able to induce the PATS (Fig. 6) suggests that the true inducer molecule would not be any of these compounds but probably a common catabolite. Taking into account that all these molecules led, through the β-oxidation pathway, to PA-CoA, which to be degraded required the activation of the genes coding for the enzymes involved in the PA catabolic pathway, we suggest that the inducer molecule for the PATS and PCL is PA-CoA. This assumption is reinforced by the absence of PATS or PCL induction when 2-phenylpropionic (α-methyl-PA) or 2-phenylbutyric acid (α-ethyl-PA), two mol-

TABLE 6. Characterization of several mutants of *P. putida* U unable to catabolize PA[a]

Mutant	PCL activity[b]	PATS (pmol min^{-1})	Bacterial growth (A_{540}) with[c]:			
			Phenylhexanoic acid	Phenyloctanoic acid	Phenylheptanoic acid	Phenylnonanoic acid
Control[d]	+	2,460	1.58 (25)	2.44 (22)	1.29 (50)	1.78 (40)
PA_1	+	ND[e]	0.66 (74)	0.97 (76)	1.32 (50)	1.68 (40)
PA_2	+	ND	0.56 (83)	0.90 (82)	1.36 (50)	1.82 (40)
PA_3	+	ND	0.64 (83)	0.94 (82)	1.21 (50)	1.72 (40)
PA_4	−	ND	0.67 (50)	1.02 (60)	1.29 (50)	1.80 (40)
PA_5	+	ND	0.63 (83)	0.96 (76)	1.22 (50)	1.74 (40)
PA_6	−	ND	0.66 (50)	0.99 (65)	1.34 (50)	1.80 (40)
PA_7	−	ND	0.87 (74)	0.99 (84)	1.11 (50)	1.69 (40)
PA_8	+	ND	0.86 (50)	1.02 (60)	1.35 (50)	1.83 (40)
PA_9	−	ND	0.89 (60)	1.04 (74)	1.51 (50)	1.86 (40)
PA_{10}	−	ND	0.89 (60)	0.94 (74)	1.47 (50)	1.91 (40)

[a] The data reported in this table are the averages of three different assays.
[b] Bacteria were grown in MM containing the antibiotics kanamycin and rifampin (12.5 and 5 μg ml^{-1}, respectively) as well as 4-hydroxy-PA (5 mM) and PA (2.5 mM) as carbon sources.
[c] An A_{540} value of 0.5 equals 5×10^8 bacteria per ml. Figures are maximal growth, and the figures in the parentheses indicate the time (in hours) when maximal growth was reached.
[d] The control was a mutant of *P. putida* U having a Tn*5* insertion in a place on the chromosome which does not affect the catabolism of PA.
[e] ND, no detection. The lowest limit of detection was 75 pmol/min (blank).

ecules that did not generate PA-CoA by β-oxidation, were supplied to cultures grown in the same conditions reported above.

It does not escape us that PA (which could be generated by hydrolysis of the endogenous PA-CoA synthesized by β-oxidation of these phenyl derivatives) could be the inducer. However, HPLC analysis of the crude extracts of *P. putida* grown in either 6-phenylhexanoic or 8-phenyloctanoic acid (5 mM) (see Materials and Methods) revealed the existence of a small peak with the retention time of PA-CoA (and which increased in size when exogenous PA-CoA was added to the sample to be analyzed), whereas no free PA was found (results not shown). We therefore suggest that because of the absence of PA in the extracts, the inducer molecule of both the PATS and PCL (and probably of the whole catabolic pathway of PA) is PA-CoA.

To establish whether the PATS which appeared when cells were grown in media containing PA or phenyl derivatives was the same or two different transport systems, the optimal physicochemical parameters and kinetic constants of the system after growth with the two inducers were compared. All the data obtained for the uptake systems induced by phenyl derivatives (optimal pH and temperature, K_m, inhibitors, and substrate specificity) were identical to those found for the PATS induced by PA, indicating that there are not two different transport systems but rather a single one. The lack of the PATS in the 10 mutants handicapped in the utilization of PA (isolated by mutagenesis with the transposon Tn*5*) (Table 6) would be irrelevant if all of them had the Tn*5* insertion in the gene (or genes) coding for the proteins involved in the PATS. However, we have shown that the 10 mutants isolated correspond to at least two different groups: mutants containing a functional PCL enzyme and those lacking it (Table 6). The induction of PCL in the mutants in the first group, even though PA could not be catabolized and the PATS was not detectable, suggests the following. (i) Although it is at a very low rate (probably by passive diffusion or by the same uptake system of 4-hydroxy-PA [see Materials and Methods]), some PA enters the cell, and this small quantity would be able to cause PCL induction. (ii) Phenylbutyrate, phenylhexanoate, and phenyloctanoate induce a transport system which could unspecifically catalyze the uptake of some PA.

The absence of the PATS in all the mutants tested (with or without PCL) (Table 6) indicates that the insertion of Tn*5* in (i) a PATS-specific gene, (ii) a different gene of the catabolic pathway, or (iii) a regulatory gene controlling the expression of the whole catabolic pathway has a common effect, namely, a lack of PA uptake. These results suggest the existence in the bacteria of a mechanism for avoiding the risk represented by the synthesis of PA-CoA, which cannot be exploited to obtain the catabolites necessary to feed the different pathways of the central metabolism because of the existence of a catabolic blockade.

In summary, in view of the foregoing, the following conclusions can be drawn: (i) PATS is a single active transport system specifically induced by PA and also, gratuitously, by certain phenyl derivatives containing an even number of carbon atoms in the aliphatic chain linked to the benzene ring; (ii) PA-CoA was identified in vivo as a true catabolite derived from PA; and (iii) PATS and PCL were identified in the bacteria cultured in minimal media containing PA or analog compounds which, throughout the general metabolism, lead to PA-CoA.

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