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Catabolism of 3- and 4-Hydroxyphenylacetate by the 3,4-Dihydroxyphenylacetate Pathway in *Escherichia coli*

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Various strains of *Escherichia coli* (but not strain K-12) were found to grow on 3-hydroxyphenylacetate and 4-hydroxyphenylacetate. Both compounds were catabolized by the same pathway, with 3,4-dihydroxyphenylacetate as a substrate for fission of the benzene nucleus, and with pyruvate and succinate as products. All the necessary enzymes were demonstrated in cell extracts prepared from induced cells but were essentially absent from uninduced cells. Mutants unable to grow on 3- and 4-hydroxyphenylacetate were defective in particular enzymes of the pathway. The characteristics of certain mutants indicated that either uptake or hydroxylation of 3- and 4-hydroxyphenylacetate may involve a common protein component. *E. coli* also grew on 3,4-dihydroxyphenylacetate, with induction of the enzymes necessary for its degradation but not those for the uptake-hydroxylation of 3- and 4-hydroxyphenylacetate.

Pseudomonads catabolize a wide range of aromatic compounds, and almost all of our present knowledge on aromatic degradation has come from studies with these organisms (6, 15). As well as delineating many catabolic sequences, such studies have also resulted in the discovery of aromatic catabolic sequences encoded in plasmids (4, 7, 16).

Although *Escherichia coli* has been used for many studies in microbial biochemistry its ability to grow on aromatic compounds has not been studied. That *E. coli* can catabolize aromatic compounds involved in the degradation of the aromatic amino acids phenylalanine and tyrosine perhaps should not be so surprising since the organism may meet such compounds in its intestinal-fecal environment. In fact, a recent paper identifying a variety of aromatic products formed by the fecal degradation of tyrosine (14) shows that such compounds are present in the normal environment of *E. coli*.

We have observed that *E. coli* is capable of utilizing certain aromatic compounds and, as described in this paper, the sequence for 4-hydroxyphenylacetate (4-HPA) catabolism appears to be identical to that already established for certain pseudomonads and species of *Acinetobacter* (1, 11, 13) (Fig. 1). However, unlike fluorescent pseudomonads (P. J. Chapman, personal communication) and *Acinetobacter* (13), *E. coli* catabolizes 3-hydroxyphenylacetate (3-HPA) and 4-HPA by the same metabolic pathway.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strains B and C were obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, Conn. *E. coli* K-12 strain K10 and *E. coli* strain W were from laboratory stocks. They were grown aerobically at 37°C in a minimal medium described previously by Hareland et al (9). Carbon sources were sterilized separately and added to give the indicated final concentrations of HPA (5 mM) or succinate (15 mM) as appropriate.

Cell-free extracts. Bacteria from 500 ml of medium were harvested in the late logarithmic phase of growth and washed with 40 ml of 0.1 M sodium-potassium phosphate buffer (pH 7.0), and the cell paste was frozen at −20°C. The frozen cells were broken in a Hughes press and extracted with 3 volumes of 0.1 M Tris-hydrochloride buffer (pH 7.8). The crude cell extracts were incubated with approximately 1 mg of crystalline DNase for 30 min at 2°C and then centrifuged at 25,000 × *g* for 15 min at 4°C to remove cell debris. The protein concentration of extracts was measured by the biuret method (8), using crystalline bovine serum albumin as standard, and was between 20 and 30 mg/ml.

Washed-cell suspensions. Cells from logarithmic-phase cultures were harvested, washed once with 0.1 M sodium-potassium phosphate buffer (pH 7.0), and finally suspended in the same buffer to give concentrations of 8 to 10 mg (dry weight) of cells per ml.

Measurement of oxygen consumption. Oxygen consumption by washed-cell suspensions or cell-free extracts was measured polarographically at 22°C using a Gilson Oxygraph, model K-1C, fitted with a Clark oxygen electrode.

Enzyme assays. The buffer used in all assays except the decarboxylation of 5-carboxymethyl-2-hydroxymuconic acid (CHMA) was 0.1 M Tris-hydro-

chloride (pH 7.8). For CHMA decarboxylase and the subsequent production of pyruvate and succinate semialdehyde the buffer was 0.1 M sodium-potassium phosphate (pH 7.5). All assays were carried out at 22°C.

Enzyme activity against 3-HPA or 4-HPA was assayed by measuring either NADH oxidation spectrophotometrically at 340 nm or oxygen consumption polarographically with a Gilson oxygen electrode system. The reaction mixture contained the following in a total volume of 1.3 ml: 0.02 ml of 0.013 M NADH and approximately 0.6 mg of crude extract protein. After measuring the blank rate the reaction was started by adding 0.01 ml of 0.02 M 3-HPA or 4-HPA.

3,4-Dihydroxyphenylacetate 2,3-dioxygenase (EC 1.13.11.15) was assayed by measuring oxygen consumption polarographically with a Gilson oxygen electrode system or spectrophotometrically by measuring the formation of 5-carboxymethyl-2-hydroxymuconic acid semialdehyde (CHMSA) at 380 nm. The rate of formation of the product was calculated from ϵ = 35,500 for CHMSA at pH 7.8 (Y-L.T. Lee, M.S. thesis, University of Minnesota, St. Paul, 1977). The reaction mixture contained 0.01 ml of 0.02 M 3,4-dihydroxyphenylacetate in a total volume of 1.3 ml, and the reaction was started by the addition of 0.05 to 0.3 mg of crude extract protein.

CHMSA dehydrogenase was assayed by measuring the decrease in absorbance at 380 nm that occurred as the substrate was oxidized. Cuvettes contained in 1.0 ml 40 to 50 nmol of CHMSA and 0.05 to 0.3 mg of crude extract protein. The reaction was started by adding 0.3 μmol of NAD. The extinction coefficient for CHMSA is given in the preceding assay.

CHMA decarboxylase was assayed by observing the decrease in absorbance at 300 nm that occurred as the substrate was transformed. The rate of decarboxylation was calculated from ϵ = 20,000 for CHMA computed from the data of Sparnins et al. (13). Cuvettes contained the following in 1.0 ml: 60 nmol of CHMA and 5 μmol of $MgSO_4$, and the reaction was started by adding 0.1 to 0.2 mg of crude extract protein.

Succinate semialdehyde dehydrogenase (EC 1.2.1.16) was assayed from the increase in absorbance at 340 nm as NAD(P) was reduced. Reaction mixtures contained 0.3 μmol of NAD(P) and 0.05 to 0.10 mg of crude extract protein in 1.0 ml, and the reaction was started by the addition of 1 μmol of succinate semialdehyde. The value ϵ = 6,200 was used to calculate the amount of NAD(P)H formed.

Isolation of mutants. To select 4-HPA-negative mutants, cultures of *E. coli* strain C were mutagenized in minimal salts medium with ethylmethanesulfonate as described by Miller (12). Survivors were allowed to grow overnight at 37°C in succinate minimal medium. Samples of these cultures were then grown on 4-HPA and treated with penicillin (12). The surviving cells were harvested and spread onto succinate plates. The colonies obtained were replica-plated onto 4-HPA plates to identify the 4-HPA-negative mutants.

Analytical methods. The 2,4-dinitrophenylhydrazones of pyruvate and succinate semialdehyde were separated by thin-layer chromatography (13); pyruvate and succinate semialdehyde were determined enzymically, as described by Sparnins et al. (13).

Chemicals. 2-, 3-, and 4-HPA, 2,5-dihydroxyphenylacetate, 3,4-dihydroxyphenylacetate, 4-aminobutyrate, crystalline DNase, crystalline lactate dehydrogenase, and crystalline bovine serum albumin were all from Sigma Chemical Co. Succinate semialdehyde was synthesized as described by Jakoby (10). CHMSA and CHMA were prepared enzymatically from 3,4-dihydroxyphenylacetate by using extracts prepared from 4-HPA-grown *Pseudomonas* U, as described by Sparnins et al. (13). Aqueous solutions of CHMSA and CHMA (approximately 5 mM concentration) were stored at −20°C.

RESULTS

Growth on hydroxyphenylacetic acids. *E. coli* strains B, C, and W, but not strain K-12, grew readily at the expense of either 3-HPA or 4-HPA. In a minimal salts medium at 37°C *E. coli* strain C grew on either 3-HPA or 4-HPA with a doubling time of 105 min. Neither *E. coli* strain C nor any of the other *E. coli* strains grew at the expense of 2-HPA.

Oxidative abilities of whole cells. When succinate-grown cells of *E. coli* strain C were tested for their ability to oxidize various aromatic compounds related to hydroxyphenylacetic acid, rates of oxidation were not significant (Table 1). However, when cells growing on succinate were exposed for 2 h to 4-HPA, the cells were induced to oxidize 3-HPA, 4-HPA, and 3-4-dihydroxyphenylacetate, but failed to oxidize 2,5-dihydroxyphenylacetate. When 3-HPA was used instead of 4-HPA in such experiments, the induction pattern was similar to that for cells grown in the presence of 4-HPA (Table 1). Cells grown on succinate in the presence of 3,4-dihydroxyphenylacetate readily oxidized that aromatic compound, but none of the other compounds tested, including 3- and 4-HPA, was oxidized (Table 1).

Evidence for the 3,4-dihydroxyphenylacetate pathway in *E. coli*. The results of the whole-cell oxidation experiments suggested that both 3- and 4-HPA were catabolized by the 3,4-dihydroxyphenylacetate pathway (Fig. 1) rather than by the 2,5-dihydroxyphenylacetate pathway (3, 5). To obtain further information about this reaction the various enzymes involved were sought in crude extracts of *E. coli* strain C.

3- and 4-HPA hydroxylase. Extracts prepared from cells grown on either 3-HPA or 4-HPA catalyzed NADH oxidation that was dependent on the presence of either 3- or 4-HPA. NADPH was not oxidized under the same conditions. For both extracts the rates with 3-HPA and 4-HPA were equivalent. The hydroxylase activity of the crude extracts was rather low (Table 2), but no activity was found in extracts prepared from cells grown on succinate, even

TABLE 1. *Rates of oxidation by washed cells of E. coli strain C*

Cells grown on succinate with:	Aromatic acid oxidized[a]				
	3-HPA	4-HPA	3,4-Dihydroxy-phenylacetate	2,5-Dihydroxy-phenylacetate	2-HPA
None	<0.1	<0.1	<0.1	<0.1	<0.1
3-HPA	58	64	70	<0.1	<0.1
4-HPA	70	54	79	<0.1	<0.1
3,4-Dihydroxy-phenylacetate	<0.1	<0.1	120	<0.1	<0.1

[a] Expressed as nanomoles of O_2 per minute per milligram (dry weight) of cells, corrected for the endogenous rate (8 nmol of O_2 per min per mg [dry weight] of cells).

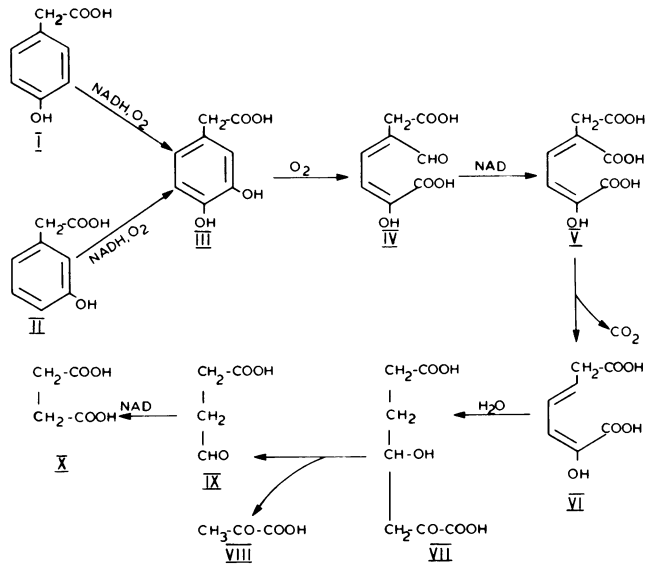

FIG. 1. *Reaction sequence for the degradation of 4-hydroxyphenylacetic acid (I) and 3-hydroxyphenylacetic acid (II) in E. coli. The intermediates are as follows: 3,4-dihydroxyphenylacetic acid (III); 5-carboxymethyl-2-hydroxymuconic acid semialdehyde (IV); 5-carboxymethyl-2-hydroxymuconic acid (V); 2-hydroxyhepta-2,4-diene-1,7-dioic acid (VI); 4-hydroxy-2-ketopimelic acid (VII); pyruvic acid (VIII); succinic acid semialdehyde (IX); succinic acid (X).*

when 3,4-dihydroxyphenylacetate was also present.

3,4-Dihydroxyphenylacetate dioxygenase. 3,4-Dihydroxyphenylacetate dioxygenase was present at high activity in extracts prepared from cells grown with either 3- or 4-HPA but could not be detected when extracts prepared from succinate-grown cells were used under the same conditions (Table 2). The spectrum of the yellow-green reaction product formed from 3,4-dihydroxyphenylacetate when measured in acid or base was identical to that expected for CHMSA (13). When 2,5-dihydroxyphenylacetate was used as substrate in the polarographic assay with extracts prepared from either 3- or 4-HPA-grown cells, there was no detectable oxygen consumption.

CHMSA dehydrogenase. CHMSA dehydrogenase was present at high activity in extracts prepared from cells grown with either 3- or 4-HPA (Table 2). When NADP was used the rate of oxidation was only 1.5% of that seen with NAD. Extracts prepared from succinate-grown cells had only 1 to 2% of the activity seen for 3- and 4-HPA-grown cells.

Formation of pyruvate and succinate semialdehyde from CHMA. Pseudomonads convert CHMA to equimolar amounts of pyruvate and succinate semialdehyde by a sequence of reactions involving decarboxylation, hydration, and aldol cleavage (13) (Fig. 1). In reaction mixtures containing 0.1 M sodium-potassium phosphate buffer (pH 7.5) and 5 mM $MgSO_4$ the extinction of CHMA monitored at 300 nm decreased very rapidly on addition of extract prepared from either 3- or 4-HPA-grown *E. coli*

strain C (Table 2). After 10 min of incubation to allow the subsequent reactions to proceed, the wavelength was changed to 340 nm, and the amount of succinate semialdehyde formed was measured as the total increase in extinction on addition of NAD due to the activity of endogenous succinate semialdehyde dehydrogenase. When NADH production had ceased more NADH was added followed by 5.5 U of crystalline lactate dehydrogenase, and the total decrease in extinction due to reduction of the pyruvate present was determined. In such experiments the amounts of succinate semialdehyde and pyruvate produced were always equivalent. The identity of these products was confirmed by chromatographic analysis of their 2,4-dinitrophenylhydrazones (13).

With a molar extinction coefficient of 2×10^4 for CHMA the amounts of succinate semialdehyde and pyruvate formed were also equivalent to the amount of CHMA added. Extracts prepared from succinate-grown cells used under the same conditions caused no decrease in extinction at 300 nm and failed to produce pyruvate. Similarly extracts prepared from cells grown on succinate with 4-aminobutyrate as nitrogen source showed no activity, even though succinate semialdehyde dehydrogenase was present in such extracts (see below).

Succinate semialdehyde dehydrogenase. Succinate semialdehyde dehydrogenase was present at high activity in extracts prepared from both 3- and 4-HPA-grown cells (Table 2), and the rate with NAD as coenzyme was almost 3 times that with NADP. Extracts prepared from succinate-grown cells had less than 4% of this activity. However, when cells were grown at 30°C on succinate with 4-aminobutyrate instead of NH_4Cl as nitrogen source succinate semialdehyde dehydrogenase was again induced and the activity with NAD (0.17 μmol min^{-1} mg $protein^{-1}$) was again almost three times higher than that with NADP.

Growth characteristics of 4-HPA-negative mutants. Mutants unable to grow on 4-HPA but still capable of growth on succinate were isolated as described in Materials and Methods. All such mutants when tested were also unable to grow on 3-HPA.

When growth on 3,4-dihydroxyphenylacetate was checked some mutants grew as readily as the parent organism, suggesting that their defect was in either the uptake or hydroxylation of 3- or 4-HPA. When extracts were prepared from such mutants grown on succinate and induced for 2 h by the further addition of 5 mM 4-HPA, no 3- or 4-HPA hydroxylase could be detected.

Other kinds of mutants could be identified by their response when patched onto plates of glycerol plus 4-HPA. One type produced a deep brown color in the agar and a second type produced a deep yellow color. It seemed likely that the mutants producing the brown color were able to form 3,4-dihydroxyphenylacetate but lacked the dioxygenase to metabolize it further, such that it accumulated and gave a dark brown oxidation product. The mutants producing the yellow color seemed likely to be defective in the dehydrogenase for CHMSA. No dioxygenase activity was detected in extracts prepared from the putative 3,4-dihydroxyphenylacetate dioxygenase mutants when grown on succinate and induced for 2 h by further addition of 5 mM 4-HPA. When the putative CHMSA dehydrogenase mutants were similarly tested no activity for the dehydrogenase could be found.

When three independent 4-HPA transport-hydroxylase mutants were spread onto 4-HPA plates and incubated for 3 to 4 days at 30°C clones appeared that had regained the ability to grow on 4-HPA. All such clones had also regained the ability to grow on 3-HPA at 30°C. When these revertants were tested at 37°C an exact equivalence in response to 3-HPA and 4-HPA was again observed in that some revertants grew on both compounds, whereas others were temperature sensitive and grew on neither. With an initial selection on 3-HPA plates the growth characteristics of the revertants obtained were exactly as those described with 4-HPA as the initial selection medium.

TABLE 2. *Induction of enzymes for 3- and 4-HPA catabolism*

Enzyme assayed[a]	Growth substrate		
	3-HPA	4-HPA	Succinate
3- or 4-HPA hydroxylase	26	28	<1
3,4-Dihydroxyphenylacetate dioxygenase	320	300	<1
CHMSA dehydrogenase	290	305	5
CHMA decarboxylase	200	190	4
Succinate semialdehyde dehydrogenase (NAD^+-linked)	260	305	8

[a] Activities expressed as nanomoles of substrate transformed per minute per milligram of protein. CHMSA, 5-carboxymethyl-2-hydroxymuconate semialdehyde; CHMA, 5-carboxymethyl-2-hydroxymuconate.

DISCUSSION

The results presented here show that in *E. coli* strain C 4-HPA is catabolized by a *meta*-cleavage route, with 3,4-dihydroxyphenylacetate

as the ring fission compound, identical to that taken by various pseudomonads and *Acinetobacter* sp. (1, 11, 13) (Fig. 1). *E. coli* strain C also appears to catabolize 3-HPA by this same route, whereas 3-HPA is hydroxylated by other organisms to give 2,5-dihydroxyphenylacetate, which is catabolized to fumarate and acetoacetate (5, 13).

This inducible pathway for 3- and 4-HPA catabolism which involves at least eight enzymes (including a permease) is by far the longest catabolic sequence yet described for *E. coli*. It will be of interest to study the genetic regulation of the sequence and to identify the number of operons involved. From the results so far obtained, the failure of the 3,4-dihydroxyphenylacetate-induced cells to oxidize 3- or 4-HPA and the absence of 3- or 4-HPA hydroxylase from such cells indicate that there are at least two regulatory groups, as has been reported for pseudomonads (1, 2). In addition, the induction of an NAD-linked succinate semialdehyde dehydrogenase by growth on 4-aminobutyrate as nitrogen source, without the formation of the CHMA decarboxylase, is a strong indication of a third regulatory group.

A distinctive aspect of the results presented here is the relationship between the initial reactions of 3- and 4-HPA catabolism. Not only are both compounds catabolized by the same route with 3,4-dihydroxyphenylacetate as the first common intermediate, but cells grown on one compound are fully induced for the catabolism of the other. Although it is possible that growth on either 3- or 4-HPA simply leads to the gratuitous synthesis of the uptake system and hydroxylase for the other compound, the results with the mutants that grow on 3,4-dihydroxyphenylacetate, but fail to grow on either 3- or 4-HPA, suggests other possible explanations.

Thus, a single protein common to both 3- and 4-HPA catabolism may be defective in such mutants. This protein may be one of the following: (i) a regulator protein controlling a single uptake system that transports both 3- and 4-HPA; (ii) a single transport system for both 3- and 4-HPA; (iii) a regulator protein controlling a single hydroxylase for both 3- and 4-HPA; (iv) a single hydroxylase acting on both 3- and 4-HPA; or (v) a common regulator gene product controlling separate operons for enzymes involved in 3- and 4-HPA catabolism. Alternatively, separate structural genes specifying distinct 3- and 4-HPA hydroxylases may be closely linked on the chromosome and a mutation in the 4-HPA hydroxylase structural gene may exert a polar effect on the 3-HPA hydroxylase if this gene is situated downstream of the 4-HPA hydroxylase gene. Further work currently underway on these mutants and on the 4-HPA hydroxylase may resolve this problem.

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