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Genetic Control of the β-Ketoadipate Pathway in *Pseudomonas aeruginosa*

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The regulation and genetic control of the β-ketoadipate pathway in *Pseudomonas aeruginosa* were investigated. The pattern of enzyme induction is apparently the same as in *P. putida*. Mutants were obtained for all but 1 of the 11 structural genes; the proximity of these genes on the chromosome was examined by transduction of the mutants with phage F116. If a group of enzymes was induced by the same compounds, the corresponding genes were closely clustered. Surprisingly, some loci-specifying enzymes not sharing a common inducer were also clustered. It is suggested that this latter finding may indicate a degree of chromosomal specialization.

The β-ketoadipate pathway for the degradation of aromatic substrates is widespread among aerobic bacteria. In those organisms which have been examined, the reactions of the pathway are mediated by inducible enzymes. Ornston (25) investigated in detail the induction patterns of the enzymes in *Pseudomonas putida* (Fig. 1). One striking feature of the regulation which operates in this species is the coordinate induction of the last two enzymes of the protocatechuate branch and the first enzyme of the common, terminal reaction sequence. This group of enzymes is product-induced by β-ketoadipate or possibly by its coenzyme A (CoA) derivative. A consequence of this pattern of regulation is that two enzymes which function only in the protocatechuate branch are synthesized gratuitously by cells grown with a compound, such as benzoate, which is metabolized through the catechol branch. Ornston (25) also demonstrated this gratuitous synthesis in two other *Pseudomonas* species (*P. aeruginosa* and *P. multivorans*), suggesting that they possessed the same regulatory control mechanism. On the other hand, it did not occur in *Hydrogenomonas eutropha* or in *Moraxella calcoacetica* (*Acinetobacter*; 2). Subsesequently, Cánovas and Stanier (4) showed that in the latter organism the regulation of the pathway is indeed different in many respects from that found in *P. putida*.

Ornston (25) suggested that in *P. putida* the enzymes of the two coordinately synthesized groups (Fig. 1) might be under the control of operons (1, 18). At the genetic level, one condition for the demonstration of an operon is that the structural genes be contiguous. Until very recently, the only pseudomonad in which genetic recombination could be studied was *P. aeruginosa* (14, 16, 19). It was, therefore, decided to investigate the regulation of the β-ketoadipate pathway in *P. aeruginosa*. At the physiological level, a detailed analysis of the induction patterns was undertaken to determine the degree of similarity with *P. putida*. At the genetic level, mutations in the structural genes were mapped by transduction with phage F116 (15) to establish whether the coordination of induction was correlated with clustering of the structural genes.

MATERIALS AND METHODS

Biological materials. The parent strain of *P. aeruginosa* ATCC 15692, here designated PRS101, was obtained from B. W. Holloway (Monash University, Clayton, Victoria, Australia); it was labeled strain 1C (14) and has been characterized taxonomically as strain 131 by Stanier, Palleroni, and Doudoroff (31). Strain PRS101 is unable to grow on *cis,cis*-muconate; a mutant, PRS104, which was able to grow on *cis,cis*-muconate (probably as a result of a permeability change), was obtained as described by Ornston (25) and was used in most of the investigation. These strains and blocked mutants derived from them are listed in Table 1. Phage F116 (15) was also obtained from B. W. Holloway.

Media. The minimal medium used was that described by Ornston and Stanier (26). Solid media were prepared by autoclaving separately 2% (w/v) Oxoid Ionagar no. 2 (Oxo Ltd., London, England) and double-strength minimal medium. When they had cooled below 50 C, the solutions were combined, the

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growth substrate was added, and plates were poured. Compounds used as growth substrates were dispensed from sterile concentrated stock solutions; acids were neutralized to *p*H 6.8 with NaOH; benzoate and succinate were autoclaved, and other compounds were sterilized by passage through a membrane filter (0.45-μm pore size; Millipore Corp., Bedford, Mass.). The compounds were supplied at the following concentrations: succinate and lactate, 20 mM; benzoate, *p*-hydroxybenzoate, and adipate, 10 mM; and other compounds, 5 mM.

Complex media were prepared with Difco products. Yeast extract (YE) agar contained YE (10 g) and agar (20 g) dissolved in minimal medium (1 liter). Nutrient yeast broth (NYB) contained nutrient broth (8 g), YE (5 g), and NaCl (8.5 g) in water (1 liter). Meat infusion agar (MIA) contained meat extract (10 g), peptone (10 g), NaCl (5 g), YE (3 g), and agar (15 g) in water (1 liter).

Maintenance of cultures. All strains were stored as frozen suspensions in sealed ampoules. These were prepared by growing the bacteria overnight at 37 C on an MIA slant. The bacterial growth was washed from the slant with sufficient NYB to give a cell concentration of 10^9 to 10^{10}/ml. Portions were added to a number of sterile glass ampoules which were then sealed aseptically, frozen rapidly in alcohol-dry ice, and stored indefinitely at −20 C.

The suspension from a thawed ampoule was added to 5 ml of NYB containing 0.5% (w/v) glucose in a tube (16 by 125 mm) fitted with a stainless-steel cap. After overnight incubation at 37 C with shaking, a drop of the suspension was added to each of a series of tubes containing the same medium. These tubes were stored at 4 C for up to 2 months; when a culture was required, one tube was incubated overnight at 37 C (subsequently referred to as an "overnight broth culture").

Growth on defined media. Cultures were grown in conical flasks with capacities five times the volumes of the contents. The flasks were incubated on a rotary shaker at 30 C. Growth was measured turbidimetrically in flasks with tubular side arms, in a Klett-Summerson colorimeter with a no. 66 filter; dilutions of a dense bacterial suspension were used to obtain a calibration curve; 100 Klett units were equivalent to about 0.3 mg (dry weight) and approximately 1.8×10^9 viable cells per ml in minimal media.

Anaerobic growth. Strain PRS104 was grown anaerobically at 30 C with lactate (20 mM) as the carbon and energy source in the minimal medium described by Ornston and Stanier (26), but with the ammonium sulfate replaced by 40 mM potassium nitrate as terminal electron acceptor and nitrogen source. Conical flasks (500 ml) with side arms attached near the top and fitted with water traps contained 360 ml of medium; the compounds to be tested as inducers were added (2 ml, 0.5 M) to the side arms. The flasks were inoculated with 40 ml of a culture, which had grown anaerobically on lactate to an optical density of 100 Klett units, and were gassed with nitrogen. After the cultures had grown for two generations, the inducers were tipped in, and incubation continued for a further 0.5 generation before the cultures were harvested. The generation time was about 8 hr.

Blocked mutants. Mutants unable to grow on one or more aromatic compounds as sole sources of carbon and energy were obtained by treating cultures with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNG; Aldrich Chemical Co., Inc., Milwaukee, Wis.) as follows. Cultures (strain PRS101 or PRS104) growing exponentially on succinate were harvested by centrifugation, washed with sterile 10 mM sodium citrate buffer (*p*H 5.5), centrifuged, and suspended in the same buffer at a density of 30 Klett units. A filter-sterilized solution of NMG was added to give a final concentration of 50 μg/ml for strain PRS101 or 200 μg/ml for strain PRS104; the suspension was incubated for 1 hr at 30 C and then was serially diluted in minimal medium; 0.1-ml portions were plated on the appropriate medium. Killing and mutant formation (as judged by colonial morphology) were unreproducible; citrate buffer alone decreased the viable count, but in other buffers tested there was no mutagenesis.

Some mutants were identified by plating on minimal medium, containing the aromatic compound at the usual concentration (5 or 10 mM) and succinate at 0.5 mM; the desired mutants (and slowly growing mutants) formed small colonies. Not all classes of mutants were obtained in this way. In later experiments, after treatment with mutagen, the bacteria were plated on 20 mM succinate agar; the resulting colonies were transferred by replica plating to solid medium containing as sole carbon and energy source an aromatic compound on which the desired mutants did not grow. The mutants were streaked on YE agar, and single colonies were picked. Mutants were kept only if they were sensitive to phage F116, grew at a reasonable rate on liquid succinate medium, and were stable to reversion (less than 1 revertant per 2×10^8 cells plated).

First, mutant lesions were classified by the compounds used for growth and, second, by the compounds accumulated after growth on 20 mM lactate in the presence of 2.5 mM aromatic compound. Catechol and protocatechuate were detected with ferrous ethylenediaminetetraacetate (EDTA) (5), β-ketoadipate by the Rothera reaction (11), and *cis,cis*-muconate and β-carboxy-*cis,cis*-muconate by their ultraviolet absorption spectra (Cary model 14 recording spectrophotometer).

Enzyme levels in the mutants were determined after growth for at least three generations on 10 mM adipate or 20 mM lactate in the presence of benzoate, *p*-hydroxybenzoate, or *cis,cis*-muconate. These inducers were added at the indicated concentrations, and the values obtained are expressed as percentages of the values given by strain PRS104, grown under the same conditions. The presence of lactate depressed the levels of enzymes of the β-ketoadipate pathway by 20 to 33%.

Preparation of transducing phage. Phage F116 was grown by adding 0.1 ml of an overnight broth culture of host bacteria and 1 ml of a phage preparation containing 5×10^4 plaque-forming units to 2 ml of melted layer agar (agar, 10 g; NYB, 1 liter), maintained at 47 C. The mixture was then layered over an MIA plate and incubated for 12 to 16 hr at 37 C. To each plate

was added 5 ml of buffer [0.01 M tris(hydroxymethyl)-aminomethane(Tris)-chloride (pH 7.4) containing 0.15 M NaCl and 0.01 M $MgSO_4$]. The plates were left at 4 C for 6 to 10 hr before the supernatant fluid was removed, clarified by centrifugation for 10 min at 3,000 × *g*, and filter-sterilized. Preparations were stable at 4 C; the yield per plate was about 3 ml of a preparation containing 2×10^{10} to 2×10^{11} plaque-forming units per ml when assayed with strain PRS101 by the layer-agar method. Phage preparations were diluted when necessary in a mixture of 1 part of NYB and 9 parts of 0.15 M NaCl containing 1 mM $MgSO_4$.

Transduction procedure. Overnight broth cultures of recipient bacteria were suspended in minimal medium to a density of 400 Klett units, equivalent to a viable count of 4×10^9 to 6×10^9 bacteria per ml on YE agar. A phage preparation (0.2 ml), containing 5×10^{10} plaque-forming units per ml, was added to 0.4 ml of bacterial suspension contained in a tube (13 by 100 mm). After 15 min of stationary incubation at 37 C, 0.1-ml portions were spread on dried plates containing the desired compound as sole carbon and energy source. Controls (recipient bacteria and phage plated separately) were performed routinely. Usually 50 to 500 colonies per plate were obtained.

Two methods were used to investigate the co-transducibility of markers on different genes; in both cases, a given mutant was used as the donor and another as the recipient. In the first method, termed "donor phenotype selection" (7), selection was made on a medium (selection medium) which allowed growth of both wild-type and donor-type recombinants; colonies were transferred with sterile toothpicks to a medium (replication medium) on which only the wild-type recombinants could grow. The percentage of co-transduction is defined as the percentage of colonies found on the selection medium that failed to grow on the replication medium. This method suffered from two limitations. First, since the structural genes being tested governed enzymes which operated in one pathway, experiments could only be performed by using as recipient the mutant blocked later in the pathway. Second, the selection medium had to contain as growth substrate a compound which was a metabolite occurring between the two enzymatic lesions being studied (or a compound which could give rise to such an intermediate by an independent branch of the pathway). Since certain such intermediates were either unavailable or could not be used as growth substrates, only certain pairs of markers could be studied by this method. Consequently, a second method was also used (12); phage preparations were made from a series of mutants, and each preparation was crossed with each mutant, in turn, as recipient. Wild-type recombinants were selected and counted; in such crosses, co-transduction is indicated by an unusually low yield of recombinants. However, there were variations both in the transducing potencies of phage preparations obtained from different mutants and in the ease with which different mutants could be transduced by a phage preparation obtained from the wild-type strain. The method, therefore, gave only qualitative information about linkage relationships; it will be termed the "qualitative method."

Manometric experiments. Cultures growing exponentially were harvested by centrifugation in a Sorvall RC-2 centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.), washed with 50 mM Na_2HPO_4-KH_2PO_4 buffer (*p*H 6.8), and suspended in the same buffer to a turbidity of 200 Klett units. Manometer flasks contained cell suspension (2 ml) in the main compartment, substrate (10 μmoles) in the side arm, and NaOH (1 mmole) in the center well. Incubations were carried out with air as the gas phase at 30 C in a differential respirometer (Gilson Medical Electronics, Middleton, Wis.).

Preparation of extracts. Cultures growing exponentially were harvested when the turbidity had reached 80 to 140 Klett units by centrifugation in the SS-34 head of a Sorvall RC 2 centrifuge for 10 min at 0 C and 7,000 × *g*. The cells were suspended in cold 20 mM Tris-chloride (*p*H 8.0), containing 10 μM MgK_2 EDTA (extraction buffer) to 20% of the original culture volume, and were centrifuged. The washed, packed cells were used immediately or stored at −15 C.

For extraction, the cells were suspended in a volume of the extraction buffer equivalent to 2% of the original culture volume. The cells were disrupted for 2.5 min with a 20-kc, 60-w ultrasonic oscillator with a ¼-inch (0.64 cm) probe operating at 1.0 amp (Measuring & Scientific Equipment, Ltd., London, England). The probe had been previously cooled, and the glass vessel containing the cells was cooled in ice water during treatment. The resulting extracts were centrifuged for 15 min at 0 C and 9,000 × *g*, and the supernatant fluid was decanted and used as crude extracts for enzyme assays; extracts were stored on ice until used.

Enzyme assays. Enzyme assays were performed in 1-cm, 3-ml silica cuvettes at 25 C. Measurements were were made with an absorbance recorder (model 2000; Gilford Instrument Laboratories, Inc., Oberlin, Ohio) connected to a Beckman DU monochromator (Beckman Instruments, Inc., South Pasadena, Calif.). Except for β-ketoadipate transferase, a unit of enzyme activity is defined as the amount of enzyme catalyzing the disappearance of 1 μmole of substrate under the conditions employed. For β-ketoadipate CoA-transferase, the unit is defined arbitrarily as the amount of enzyme required to cause a change of 1.0 absorbance unit per min under the assay conditions employed.

Published assay methods were used for catechol oxygenase (catechol:oxygen 1,2-oxidoreductase), EC 1.13.1.1 (13); *cis,cis*-muconate lactonizing enzyme (4 - carboxymethyl - 4 - hydroxyisocrotonolactone lyase (decyclizing)), EC 5.5.1.1 (24); muconolactone isomerase (24); *p*-hydroxybenzoate hydroxylase (17); protocatechuate oxygenase (protocatechuate:oxygen 3,4-oxidoreductase), EC 1.13.1.3 (30); carboxymuconate lactonizing enzyme and carboxymuconolactone isomerase (23); enol-lactone hydrolase [incorrectly listed (23) as carboxymethyl-4-hydroxyisocrotonolactone hydrolase, EC 3.1.1.16]; β-ketoadipate CoA-transferase (3-ketoadipate succinyl-CoA transferase), EC 2.8.3.6 (4). These assays were satisfactory for *P. aeruginosa; p*H optima and Michaelis constants were, therefore, not determined.

Estimation of protein. The method of Lowry et al. (20) was used to determine protein concentrations with bovine plasma albumin (Fraction V, Armour Pharmaceutical Co., Kankakee, Ill.) as a standard.

Reagents. Intermediates of aromatic acid catabolism which were unavailable commercially were prepared as described by MacDonald and Stanier (21). Succinyl-CoA was prepared by the method of Stadtman (28). Purified enzymes, which were required for some of the enzyme assays and were obtained by the methods of Ornston (23, 24), were kindly supplied by members of the Department.

RESULTS

Ornston (25) clearly established for the β-ketoadipate pathway in *P. putida* the reaction steps and induction patterns (Fig. 1). From his preliminary observations, he suggested that the same pathway and induction patterns occurred in *P. aeruginosa*, and the present experiments were planned on that assumption. In addition to the enzymes studied by Ornston in *P. putida*, *p*-hydroxybenzoate hydroxylase, β-ketoadipate CoA-transferase, and the oxidation of benzoate and anthranilate to catechol were also studied in *P. aeruginosa* (Fig. 2). The nature of the inducer of each enzyme was investigated in detail with the aid of blocked mutants (Tables 1 and 2).

Catechol branch: benzoate and anthranilate oxidases. It has thus far proved impossible to assay satisfactorily the enzymes involved in the conversion of benzoate or anthranilate to catechol (hereafter termed the benzoate and anthranilate oxidase systems). Evidence for the inducers of these systems was obtained from manometric experiments (Table 3). Each system was induced by its substrate; there was no induction by catechol or subsequent intermediates.

Catechol branch: catechol oxygenase, muconate lactonizing enzyme, and muconolactone isomerase. Extracts of strain PRS136, which were unable to convert benzoate to catechol, had only uninduced levels of the subsequent enzymes when prepared from cells grown in the presence of benzoate (Table 4). Hence, benzoate cannot directly induce enzymes of the pathway except the benzoate oxidase system, and the synthesis of the benzoate oxidase system represents a sequential induction (29). Catechol oxygenase was induced not only when strain PRS104 was grown on catechol but also gratuitously when it was grown on *cis,cis*-muconate (Table 5). The irreversible nature of the reaction made it unlikely that the latter acted as an inducer by conversion to catechol, and the possibility that catechol acted directly as an inducer was eliminated by growing *P. aeruginosa* anaerobically with nitrate as the terminal electron acceptor; under these conditions, reactions involving molecular oxygen cannot occur, and catechol is not converted to *cis,cis*-muconate. When strain PRS104 was grown anaerobically on lactate and catechol or *cis,cis*-muconate was added (Table 6), only the latter acted as an inducer; toxicity of catechol was eliminated as an explanation by a control in which both compounds were added. Hence, catechol oxygenase is induced not by its substrate, catechol, but by its product, *cis,cis*-muconate.

From the anaerobic growth experiment, and also from the results obtained with strain PRS121 which lacked catechol oxygenase (Table 4), it was

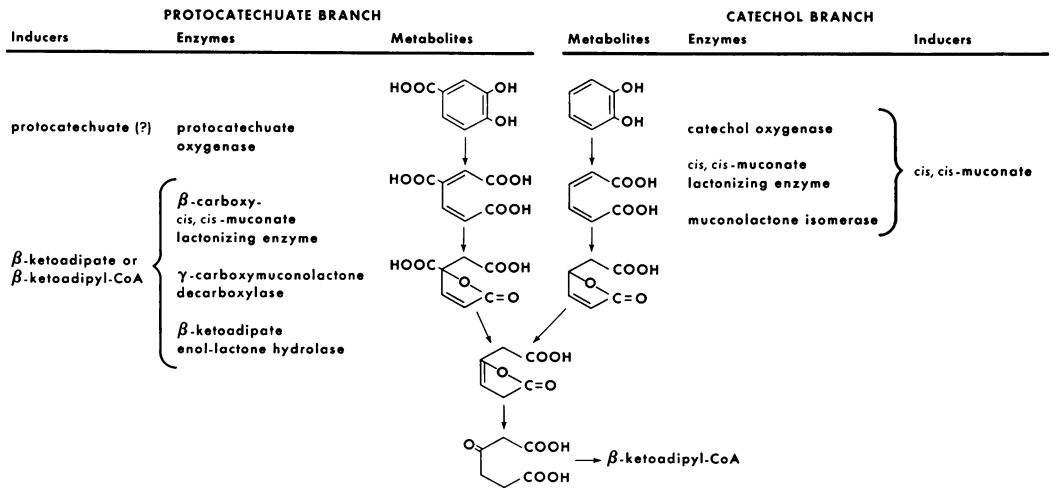

FIG. 1. *Induction pattern of enzymes of the β-ketoadipate pathway in P. putida studied by Ornston (25). Enzymes, the names of which are enclosed in brackets, are synthesized coordinately.*

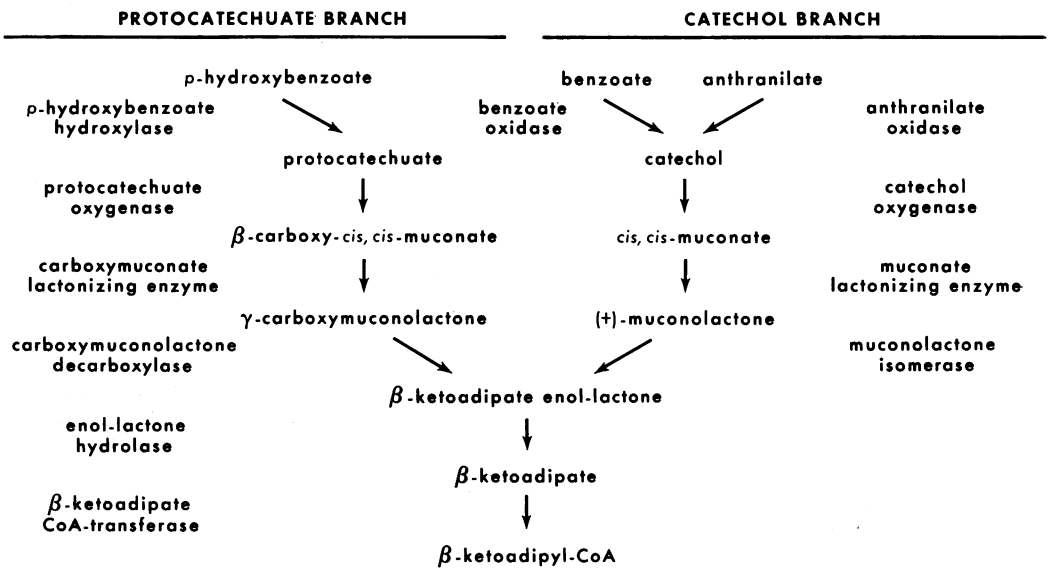

FIG. 2. *Reactions of the β-ketoadipate pathway studies in P. aeruginosa.*

TABLE 1. *Description of strains*[a]

Strain	Genotype[b]	Origin	Phenotype
PRS101		B. W. Holloway[c]	Wild
PRS104	*per*-1501	PRS101	Obtained by direct selection without mutagenesis: grows on *cis,cis*-muconate
PRS111	*catB1502 per-1501*	PRS104	Muconate lactonizing enzyme
PRS112	*catC1503 per-1501*	PRS104	Muconolactone isomerase
PRS121	*catA1504 per-1501*	PRS104	Catechol oxygenase
PRS126	*pcaE1505 per-1501*	PRS104	β-Ketoadipate CoA-transferase
PRS128	*pcaD1506 per-1501*	PRS104	Enol-lactone hydrolase
PRS133	*pcaD1507 per-1501*	PRS104	Enol-lactone hydrolase
PRS136	*ben-1508 per-1501*	PRS104	Benzoate oxidase system[d]
PRS408	*pcaB1514 per-1501*	PRS104	Carboxymuconate lactonizing enzyme
PRS413	*pobA1515 per-1501*	PRS104	*p*-Hydroxybenzoate hydroxylase
PRS416	*pcaA1516 per-1501*	PRS104	Protocatechuate oxygenase
PRS419	*ant-1517 per-1501*	PRS104	Anthranilate oxidase[d] system
PRS202	*pobA1509*	PRS101	*p*-Hydroxybenzoate hydroxylase
PRS213	*pcaB1510*	PRS101	Carboxymuconate lactonizing enzyme
PRS214	*pcaB1511*	PRS101	Carboxymuconate lactonizing enzyme
PRS215	*pcaA1512*	PRS101	Protocatechuate oxygenase
PRS228	*pcaE1513*	PRS101	β-Ketoadipate CoA-transferase

[a] All strains except PRS101 and PRS104 are "blocked mutants" obtained by NMG treatment, and exhibited the indicated enzyme deficiency.

[b] The genotype designations conform to the suggestions of Demerec et al. (10).

[c] The origin of strain PRS101 is given in Materials and Methods.

[d] If the benzoate and anthranilate oxidase systems contain more than one component enzyme or polypeptide, the mutants could be lacking one component or all of them.

concluded that catechol also did not induce the next two enzymes, muconate lactonizing enzyme and muconolactone isomerase. (±)-Muconolactone, offered to cells growing on lactate (Table 5), also failed to induce any enzymes measured. Muconolactone is able to enter the cells, however, since the (+) isomer is oxidized by cells grown on *cis,cis*-muconate. Failure to serve as an inducer of the requisite enzymes would explain why strain PRS104 fails to grow at the expense of (+)- or (±)-muconolactone.

Since growth of strain PRS104 on *p*-hydroxy-

TABLE 2. *Enzymes, inducers, and genes of the β-ketoadipate pathway in P. aeruginosa*

Enzyme	Inducer	Gene designation[a]
p-Hydroxybenzoate hydroxylase	*p*-Hydroxybenzoate	*pobA*
Protocatechuate oxygenase	*p*-Hydroxybenzoate or protocatechuate or both	*pcaA*
Carboxymuconate lactonizing enzyme	β-Ketoadipate[b]	*pcaB*
Carboxymuconolactone decarboxylase	β-Ketoadipate[b]	*pcaC*
Enol-lactone hydrolase	β-Ketoadipate[b]	*pcaD*
β-Ketoadipate CoA-transferase	β-Ketoadipate[b]	*pcaE*
Benzoate oxidase system	Benzoate	*ben*⁻
Anthranilate oxidase system	Anthranilate	*ant*⁻
Catechol oxygenase	*cis,cis*-Muconate	*catA*
Muconate lactonizing enzyme	*cis,cis*-Muconate	*catB*
Muconolactone isomerase	*cis,cis*-Muconate	*catC*

[a] The gene designations conform to the suggestions of Demerec et al. (10). The benzoate and anthranilate oxidase systems have not been purified to any extent, and each may well contain more than one component polypeptide. The other enzymes have all been purified from fluorescent pseudomonads, and it is thought that no more remain to be discovered in the conversions of *p*-hydroxybenzoate and catechol to β-Ketoadipyl-CoA. It has not been possible to examine complementation in *P. aeruginosa*, and thus far studies of purified proteins have not revealed different component polypeptide chains. Therefore, the gene designations have been allotted by the one gene-one enzyme principle.

[b] β-Ketoadipyl-CoA and β-ketoadipate may both be able to act as inducers.

benzoate did not induce either muconate lactonizing enzyme or muconolactone isomerase (Table 5), the only inducer for the two enzymes must be *cis,cis*-muconate. By use of strain PRS111, which lacked muconate lactonizing enzyme, induction of muconolactone isomerase by *cis,cis*-muconate was shown directly (Table 4).

Protocatechuate branch: p-hydroxybenzoate hydroxylase and protocatechuate oxygenase. *p*-Hydroxybenzoate hydroxylase was induced when strain PRS104 was grown on *p*-hydroxybenzoate but not when grown on protocatechuate (Table 5); it is, therefore, induced only by its substrate. The second enzyme, protocatechuate oxygenase, was induced when strain PRS104 was grown on protocatechuate (Table 5), but it was also induced by *p*-hydroxybenzoate in strain PRS202, which lacked *p*-hydroxybenzoate hydroxylase and was, therefore, unable to convert *p*-hydroxybenzoate to protocatechuate (Table 7). It was concluded that both protocatechuate and *p*-hydroxybenzoate could induce protocatechuate oxygenase. Further evidence was obtained by growing strain PRS104 anaerobically in the presence of the two compounds (Table 8); *p*-hydroxybenzoate induced both enzymes, although no protocatechuate was formed, and the latter induced only protocatechuate oxygenase. This same pattern of regulation has been found in *P. putida* (K. Hosokawa, *personal communication*).

Protocatechuate branch: carboxymuconate lactonizing enzyme, carboxymuconolactone decarboxylase, and enol-lactone hydrolase. Ornston (25) showed that when *P. aeruginosa* was grown on benzoate, the three enzymes, carboxymuconate lactonizing enzyme, carboxymuconolactone decarboxylase, and enol-lactone hydrolase, were all induced; he suggested, by analogy with *P. putida*, that they were all induced by the final product, β-ketoadipate. His findings have been confirmed with strain PRS104, grown on benzoate and β-ketoadipate (Table 5). Further evidence for this striking induction pattern was obtained with two mutants. Strain PRS133, which lacked enol-lactone hydrolase, was unable to form β-ketoadipate from benzoate; when grown in the

TABLE 3. *Oxidative capacities of strain PRS104 grown on various compounds*

Substrate	Oxygen uptake at 30 C by washed suspensions of cells grown on[a]				
	Benzoate	Anthranilate	Catechol	*cis,cis*-Muconate	Lactate
Benzoate[b]	300	22	13	18	13
Anthranilate	35	170	13	18	13
Catechol[b]	350	340	175	450	22
cis,cis-Muconate	95	70	30	90	13
None (endogenous)	22	22	10	18	13

[a] Expressed as microliter per hour per milligram (dry weight).

[b] The rates with benzoate and catechol decreased sharply after the consumption of about 1 mole of oxygen per mole of substrate supplied.

TABLE 4. *Enzyme levels in extracts prepared from mutants lacking enzymes of the catechol branch, grown on lactate (20 mM) in the presence of benzoate or cis,cis-muconate (5 mM)*[a]

Enzyme	Benzoate		*cis,cis*-Muconate	
	PRS136 (lacking benzoate oxidase system)	PRS121 (lacking catechol oxygenase)	PRS111 (lacking muconate lactonizing enzyme)	PRS112 (lacking muconolactone isomerase)
Catechol oxygenase	<0.2	<0.2	52	58
Muconate lactonizing enzyme	<0.2	0.7	<0.5	89
Muconolactone isomerase	<0.1	5	85	<0.5
Enol-lactone hydrolase	3	3	3	3

[a] Activities are expressed as percentages of those obtained with strain PRS104, grown under the same conditions.

TABLE 5. *Enzyme levels in extracts prepared from strain PRS104 grown on various substrates*

Enzyme	Specific activities in extracts from cells grown on[a]									
	Benzoate	Anthranilate	Catechol	*cis,cis*-Muconate	(±)-Muconolactone and lactate	*p*-Hydroxybenzoate	Protocatechuate	β-Ketoadipate	Adipate	Lactate
Catechol oxygenase	450	460	250	560	<0.2	1	1	<1	<0.5	<0.2
Muconate lactonizing enzyme	340	380	240	330	<0.2	<0.2	<0.2	<1	<0.5	<0.2
Muconolactone isomerase	1,800	2,410	1,660	2,060	<2	10	10	<10	<5	<2
p-Hydroxybenzoate hydroxylase	20					340	2			<1
Protocatechuate oxygenase	<3					370	270			<3
Carboxymuconate lactonizing enzyme	740	730	540	580	17	560	500	610	810	10
Carboxymuconolactone decarboxylase	2,030	1,860	1,880	1,300	33	1,780	1,570	1,740	2,310	<4
Enol-lactone hydrolase	730	710	680	550	29	510	470	700	860	12
β-Ketoadipate CoA-transferase	1,000	820	700	670	<30	850	810	860	1,270	<30

[a] All levels have been multiplied by 10^3 for comparative purposes.

presence of benzoate, the three enzymes were not induced (Table 9). On the other hand, strain PRS126, which lacked the next enzyme, β-ketoadipate CoA-transferase, did accumulate β-ketoadipate when it was grown with benzoate, and all three enzymes were induced.

These three enzymes were also induced when *P. aeruginosa* was grown on adipate. The pathway of adipate catabolism in pseudomonads is not fully understood, but it seems probable that it joins the pathways of benzoate and *p*-hydroxybenzoate catabolism at β-ketoadipyl-CoA. If so, the production of the three enzymes when strain PRS126, which lacked β-ketoadipate CoA-transferase, was grown on adipate (Table 9) could be explained most simply if the CoA derivative and free β-ketoadipate could both act as inducers. There are, however, other possible explanations; the simplest is that adipate acts as a structural analogue of β-ketoadipate.

Protocatechuate branch: β-ketoadipate CoA-transferase. The results obtained with strain PRS133, which lacked enol-lactone hydrolase, showed that β-ketoadipate CoA-transferase is not induced by any compound before β-ketoadipate (Table 9); it may, however, be induced by free β-ketoadipate, the CoA ester, or both.

Protocatechuate branch: genetic experiments.

No mutants were obtained in which the structural gene for carboxymuconolactone decarboxylase (*pcaC*) was affected. The donor phenotype selection method was used to determine co-transducibility of markers in other genes of the protocatechuate branch (Table 10). The gene designations are listed in Table 2. Close linkage of gene *pcaB* to *pcaD* and *pcaE* was found; close linkage of genes *pcaD* and *pcaE* is, therefore, inferred but could not be shown directly with this technique. However, close linkage of *pcaD* and *pcaE* was demonstrated (Table 12) by the qualitative method. By this method also (Table 11), it was shown that transduction between two independent mutants (strains PRS213 and PRS214) blocked in the same enzymatic step gave the expected very low number of recombinants and, from the crosses of these mutants with strain PRS228, close linkage of genes *pcaB* and *pcaE* was confirmed. By the donor phenotype selection method, a marker in gene *pobA* was co-transducible, at relatively low frequencies, with markers in the *pcaB-pcaD-pcaE* group (Table 10); on the other hand, a marker in gene *pcaA* was not co-transducible with any other markers tested.

TABLE 6. *Induction of enzymes by catechol and cis,cis-muconate supplied to strain PRS104 growing anaerobically on lactate*

Enzyme	Specific activity in extracts of cells grown in the presence of[a]			
	Catechol	*cis,cis*-Muconate	Both	Neither
Catechol oxygenase	<0.4	21	13	<0.2
Muconate lactonizing enzyme	<0.2	105	86	<0.2
Muconolactone isomerase	<2	450	530	<2

[a] All levels have been multiplied by 10^3 for comparative purposes.

Catechol branch: genetic experiments. The results of a donor phenotype selection experiment (Table 13) showed more or less close linkage of genes *ben*$^-$, *ant*$^-$, *catA*, *catB*, and *catC*, but there was no co-transduction of markers in any of the above genes with markers in genes *pcaD* or *pcaE*. Close linkage of genes *catB* and *catC*, which could not be shown by the donor phenotype selection method, was shown by the qualitative method (Table 12).

DISCUSSION

No differences were found between the patterns of induction of the enzymes of the β-ketoadipate

TABLE 7. *Enzyme levels in extracts prepared from strains PRS203, PRS215, and PRS214 grown on lactate (20 mM) in the presence of p-hydroxybenzoate (10 mM)*[a]

Enzyme	PRS202 (lacking *p*-hydroxybenzoate hydroxylase)	PRS215 (lacking protocatechuate oxygenase)	PRS214 (lacking carboxymuconate lactonizing enzyme)
p-Hydroxybenzoate hydroxylase	<0.5	20	17
Protocatechuate oxygenase	63	<1	15
Carboxymuconate lactonizing enzyme	3	3	<0.5
Carboxymuconolactone decarboxylase			<1
Enol-lactone hydrolase	2	2	4
β-Ketoadipate CoA-transferase			<5

[a] Activities are expressed as percentages of those obtained with strain PRS104 grown under the same conditions. Mutants lacking protocatechuate oxygenase or carboxymuconate lactonizing enzyme and grown in the presence of *p*-hydroxybenzoate accumulated intermediates and grew poorly; this may account for the low enzyme levels.

TABLE 8. *Induction of enzymes by p-hydroxybenzoate and protocatechuate supplied to cells of strain PRS104 growing anaerobically on lactate*

Enzyme	Specific activity in extracts from cells grown in the presence of[a]		
	p-Hydroxybenzoate	Protocatechuate	Neither
p-Hydroxybenzoate hydroxylase	24	<1	<1
Protocatechuate oxygenase	50	220	<1
Carboxymuconate lactonizing enzyme	10^b	10^b	10^b

[a] All levels have been multipled by 10^3 for comparative purposes.
[b] This is the basal level expected from aerobically grown cells (Table 5).

TABLE 9. *Enzyme levels in mutant strains PRS133 and PRS126 grown in basal medium supplemented with various compounds*[a]

Enzyme	PRS133 (lacking enol-lactone hydrolase)		PRS126 (lacking β-ketoadipate CoA-transferase)	
	cis,cis-Muconate (5 mM) + lactate (20 M)	Adipate (10 mM)	*cis,cis*-Muconate (5 mM) + lactate (20 mM)	Adipate (10 mM)
Muconolactone isomerase	80		130	
Carboxymuconate lactonizing enzyme	3	165	460	120
Carboxymuconolactone decarboxylase	<1	185	360	138
Enol-lactone hydrolase	<1	<1	400	124
β-Ketoadipate CoA-transferase	<5	120	<5	<5

[a] Activities are expressed as percentages of those obtained with strain PRS104 grown under the same conditions.

TABLE 10. *Donor phenotype selection method—protocatechuate branch*

Donor		Recipient		Selection[a] medium	No. of colonies examined	Co-transduction (%)
Strain	Genotype	Strain	Genotype			
PRS413	*pobA*	PRS416	*pcaA*	Protocatechuate	100	0
PRS413	*pobA*	PRS408	*pcaB*	Protocatechuate	200	39
PRS413	*pobA*	PRS133	*pcaD*	Benzoate	100	43
PRS416	*pcaA*	PRS133	*pcaD*	Benzoate	100	0
PRS408	*pcaB*	PRS133	*pcaD*	Benzoate	100	98
PRS413	*pobA*	PRS126	*pcaE*	Benzoate	300	22
PRS416	*pcaA*	PRS126	*pcaE*	Benzoate	100	0
PRS408	*pcaB*	PRS126	*pcaE*	Benzoate	200	89

[a] Colonies were replicated to *p*-hydroxybenzoate plates.

TABLE 11. *Transduction frequencies between mutants—protocatechuate branch*

Recipient		No. of transductants obtained per plate[a] using phage grown on strain (genotype in parentheses)						
Strain	Genotype	PRS202 (*pobA*)	PRS215 (*pcaA*)	PRS214 (*pcaB*)	PRS213 (*pcaB*)	PRS228 (*pcaE*)	PRS101 (wild)	No phage
PRS202	*pobA*	0	190	73	96	81	60	0
PRS215	*pcaA*	124	0	158	131	97	61	0
PRS214	*pcaB*	37	110	0	0	10	35	0
PRS213	*pcaB*	11	76	1	0	4	21	0
PRS228	*pcaE*	61	200	22	23	0	30	1
NR[b]		0	0	0	0	0	0	

[a] Portions (0.2 ml) of transduced cultures were spread on *p*-hydroxybenzoate plates.
[b] NR, no recipient.

pathway in *P. aeruginosa* and in *P. putida* (25; K. Hosokawa, *personal communication*). These two pseudomonads, which both belong to the fluorescent group, share many phenotypic properties (31). However, they differ in the pattern of induction and number of the enzymes which convert mandelate to benzoate (S. L. Rosenberg, *in preparation*). Ornston (25) examined the effects of various catabolite repressors on the synthesis, in *P. putida*, of those enzymes which shared common inducers and found two groups whose component enzymes were synthesized coordinately (Fig. 1). In *P. aeruginosa*, it proved impossible to demonstrate any significant differences in the sensitivity to catabolite repression of the synthesis of the various enzymes, even of those which were induced

TABLE 12. *Transduction frequencies between mutants—catechol branch*

Recipient		No. of transductants obtained per plate[a] using phage grown on strain (genotypes in parentheses)							
Strain	Genotype	PRS136 (*ben*⁻)	PRS121 (*catA*)	PRS111[b] (*catB*)	PRS112[b] (*catC*)	PRS128 (*pcaD*)	PRS126 (*pcaE*)	PRS101 (wild)	No phage
PRS136	*ben*⁻	0	54	6	11	480	602	1,162	0
PRS121	*catA*	47	0	3	1	439	409	784	1
PRS111	*catB*	17	0	0	0	373	329	558	0
PRS112	*catC*	23	5	0	0	259	368	458	1
PRS128	*pcaD*	116	73	54	94	0	18	157	0
PRS126	*pcaE*	256	245	94	210	10	0	261	0
NR[c]	NR[c]	0	1	0	0	0	0	0	

[a] Portions (0.1 ml) of transduced cultures were spread on benzoate plates.
[b] Inoculum: 2×10^{10} per ml.
[c] NR, no recipient.

TABLE 13. *Donor phenotype selection—catechol branch*

Donor		Recipient		Selection medium	Replication medium	No. of colonies examined	Cotransduction (%)
Strain	Genotype	Strain	Genotype				
PRS136	*ben*⁻	PRS419	*ant*⁻	Anthranilate	Benzoate	300	84
PRS419	*ant*⁻	PRS136	*ben*⁻	Benzoate	Anthranilate	100	92
PRS136	*ben*⁻	PRS121	*catA*	Anthranilate	Benzoate	100	77
PRS419	*ant*⁻	PRS121	*catA*	Benzoate	Anthranilate	100	97
PRS136	*ben*⁻	PRS111	*catB*	*cis,cis*-Muconate	Benzoate	200	80
PRS419	*ant*⁻	PRS111	*catB*	*cis,cis*-Muconate	Anthranilate	130	91
PRS121	*catA*	PRS111	*catB*	*cis,cis*-Muconate	Benzoate	200	97
PRS136	*ben*⁻	PRS112	*catC*	*cis,cis*-Muconate	Benzoate	200	72
PRS121	*catA*	PRS112	*catC*	*cis,cis*-Muconate	Benzoate	200	99
PRS136	*ben*⁻	PRS133	*pcaD*	*p*-Hydroxybenzoate	Benzoate	100	0
PRS121	*catA*	PRS133	*pcaD*	*p*-Hydroxybenzoate	Benzoate	100	0
PRS111	*catB*	PRS133	*pcaD*	*p*-Hydroxybenzoate	Benzoate	50	0
PRS112	*catC*	PRS133	*pcaD*	*p*-Hydroxybenzoate	Benzoate	50	0
PRS136	*ben*⁻	PRS126	*pcaE*	*p*-Hydroxybenzoate	Benzoate	83	0
PRS419	*ant*⁻	PRS126	*pcaE*	*p*-Hydroxybenzoate	Anthranilate	50	0
PRS121	*catA*	PRS126	*pcaE*	*p*-Hydroxybenzoate	Benzoate	100	0
PRS111	*catB*	PRS126	*pcaE*	*p*-Hydroxybenzoate	Benzoate	40	0
PRS112	*catC*	PRS126	*pcaE*	*p*-Hydroxybenzoate	Benzoate	54	0

by different compounds. Ornston (25) suggested that in *P. putida* the coordinate groups of enzymes were controlled by operons. In an operon, mutants of a class termed "polar" (1) can be obtained; it would be desirable to search for such mutants in *P. aeruginosa*.

A further necessary, but not sufficient, condition for the demonstration of an operon is that the structural genes should be contiguous on the chromosome. For those groups of enzymes which share common inducers (Tables 10–13), the genes are very closely linked (*catA*, *catB*, and *catC*; *pcaB*, *pcaD*, and *pcaE*). In *P. putida*, Ornston (25) observed that the synthesis of catechol oxygenase was not coordinate with that of muconate lactonizing enzyme and muconolactone isomerase, but, in *P. aeruginosa*, the genes which code for all three enzymes were very closely linked. It could be that in *P. aeruginosa*, unlike *P. putida*, the three enzymes are, in fact, synthesized coordinately; on the other hand, examples are known from *Escherichia coli* in which the genes are clustered but the enzymes, although participating in the same pathway and controlled by the same co-repressor, are not synthesized coordinately (33).

An unexpected result was the clustering of certain genes which code for enzymes whose synthesis is evoked by different inducers; for example, a marker in gene *pobA* was co-transducible with markers in genes *pcaB*, *pcaD*, and *pcaE* (Table 10). Because of the size of the samples, not too

much significance should be attributed to the numerical values of the co-transduction frequencies. It seems, however, that while the genes of the *pcaB-pcaD-pcaE* group may well be contiguous (as the operon model requires), gene *pobA* is not immediately adjacent to the group. It may be that there is some overall loose clustering of the genes involved in aromatic acid catabolism in *P. aeruginosa;* if this is so, the clusters must be longer than the fragment of the chromosome which can be transduced by phage F116, since, for example, a marker in gene *pcaA* was not co-transducible with any other markers studied (Table 10). The basis of this chromosomal specialization is not clear, but it may reflect some feature of the ontogeny of the pathway and the regulatory apparatus governing its expression.

This investigation was confined to presumed structural gene mutants. The operon model includes two regulatory genes, the operator gene, which must be closely linked to the structural genes, and the repressor gene, which may, but need not, be closely linked. It is difficult to classify mutations in regulatory genes in terms of the operon model unless dominance can be determined; thus far this is impossible in *P. aeruginosa.* However, the present study is complemented by the recent investigation of Brammer, Clarke, and Skinner (3), who showed by transduction that the structural gene for the inducible enzyme amidase is very closely linked to markers which conferred constitutivity or altered inducibility.

In *Salmonella typhimurium* (9) and in *E. coli* (32), the genes which code for several enzymes of a metabolic pathway are often clustered on the chromosome. In contrast, Fargie and Holloway (12) examined a large number of auxotrophs of *P. aeruginosa* and concluded that such clustering of functionally related genes was very rare. In more detailed studies of individual pathways, Pearce and Loutit (27) and Mee and Lee (22) showed that although such clusters did occur in *P. aeruginosa* they were less common and extensive than in *E. coli* or *S. typhimurium.* Their results suggested that the regulation of biosynthetic pathways might be quite different in pseudomonads. Crawford and Gunsalus (8) investigated the biosynthesis of tryptophan by *P. putida* and found that the enzymes were synthesized in three coordinate groups. Very recently, Chakradbarty, Gunsalus, and Gunsalus (6) obtained a transducing phage for this species and demonstrated three clusters of genes corresponding to the three groups of enzymes. Furthermore, in agreement with our own results, clusters of genes were observed in two catabolic pathways in which the synthesis of the enzymes was known to be coordinate.

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