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Regulation of the β-Ketoadipate Pathway in *Alcaligenes eutrophus*

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The regulation of the synthesis of the inducible enzymes that mediate the reactions of the β-ketoadipate pathway in *Alcaligenes eutrophus* has been examined by determining the inductive responses of the wild type and of mutants derived from it to metabolites of the pathway. The system of control differs in many respects from those which operate in the genera *Pseudomonas* and *Acinetobacter*.

The aerobic dissimilation of benzoate and *p*-hydroxybenzoate by bacteria frequently proceeds through the parallel, convergent catechol and protocatechuate branches of the β-ketoadipate pathway (Fig. 1). This pathway operates in many bacterial genera, including *Pseudomonas* (14), *Acinetobacter* (6), *Azotobacter* (9), and the *Mycobacterium-Nocardia* group (2). In all organisms so far examined, the enzymes of the pathway are inducible, their synthesis being elicited by primary substrates or metabolic intermediates in the pathway. The complete pattern of induction of the enzymes that convert benzoate and *p*-hydroxybenzoate to β-ketoadipyl-CoA has been determined for three bacterial species: *Pseudomonas putida* (12), *P. aeruginosa* (11), and *Acinetobacter calcoaceticus* (3, 4, 6, 7).

The system of regulation in the two *Pseudomonas* species is identical. One of its characteristic features, also detected in other species of *Pseudomonas* (12), is the coordinate synthesis of four enzymes, β-carboxymuconate lactonizing enzyme, γ-carboxymuconolactone decarboxylase, β-ketoadipate enol-lactone hydrolase (EC 3.1.1.16, 4-carboxy-methyl-4-hydroxyisocrotonolactone hydrolase), and β-ketoadipate succinyl-CoA transferase (EC 2.8.3.6, 3-ketoadipate-succinyl-CoA transferase) of which the inducer (β-ketoadipate or β-ketoadipyl-CoA) is a metabolite common to both branches of the pathway. However, two of the enzymes (β-carboxymuconate lactonizing enzyme and γ-carboxymuconolactone decarboxylase) catalyze reactions specific to the protocatechuate branch and are thus synthesized gratuitously when *Pseudomonas* species are grown with substrates dissimilated through the catechol branch of the pathway (for example, benzoate).

The observation that β-carboxymuconate lactonizing enzyme and γ-carboxymuconolactone decarboxylase are not induced in benzoate-grown cells of *A. calcoaceticus* (12) provided the first indication that the pathway in this organism is subject to a different system of regulation; a subsequent detailed analysis (6) confirmed the existence of completely different control systems in *Acinetobacter* and *Pseudomonas*. In the light of these findings, it has been proposed that the system of regulation governing a dissimilatory pathway of wide biological distribution may be a complex, group-specific character of considerable evolutionary and taxonomic significance (5). *Alcaligenes eutrophus* provides a good biological object for testing this hypothesis, since it dissimilates benzoate and *p*-hydroxybenzoate through the β-ketoadipate pathway (10) but belongs to a taxonomic group distinct from both *Pseudomonas* and *Acinetobacter* (1, 8).

Ornston (12) demonstrated that β-carboxymuconate-lactonizing enzyme and carboxymuconolactone decarboxylase are not induced in benzoate-grown cells of *A. eutrophus*. We have confirmed this finding and observed that induction of the enzymes operative in the parallel convergent sequences leading from benzoate and *p*-hydroxybenzoate to β-ketoadipate enol-lactone is physiologically specific, i.e., none of the enzymes involved in benzoate metabolism is significantly induced in *p*-hydroxybenzoate-grown cells, and vice versa (10). As we shall show here, the regulatory system governing the synthesis of the enzymes of the β-ketoadipate pathway in *A. eutrophus* is unique and differs in many respects from the regulatory systems that operate in *Pseudomonas* and *Acinetobacter*.

MATERIALS AND METHODS

A. eutrophus 335 (ATCC 17697) and mutants derived from it were used. The media and methods of cultivation as well as the procedures used to prepare cell-

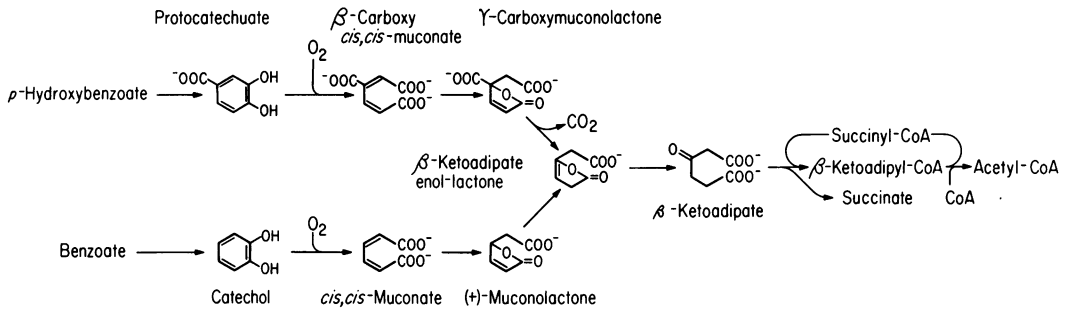

FIG. 1. *Chemistry of the β-ketoadipate pathway.*

free extracts and to perform enzyme assays have been described (10).

Mutagenesis and selection of mutants. Mutants unable to utilize aromatic compounds as carbon and energy sources were induced by treatment of *A. eutrophus* 335 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and were enriched by growth on an aromatic compound in the presence of penicillin G and D-cycloserine. A culture of *A. eutrophus* 335 growing exponentially on succinate was harvested by centrifugation, washed aseptically with tris(hydroxymethyl)aminomethane-malate buffer (*p*H 5.5), centrifuged, and resuspended in the same buffer. The suspension, containing 10^8 cells/ml and 200 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml, was incubated for 2 hr at 30 C without shaking. Samples of cells (harvested by centrifugation) were added to 25 ml of mineral medium containing 1 mM succinate and allowed to grow for several generations for mutations to achieve phenotypic expression. Mutants unable to grow at the expense of a particular aromatic compound were then enriched by placing samples of the culture in a medium containing the aromatic compound in question (concentration, 2.5 mM), together with 500 μg of D-cycloserine per ml and 2,000 units of penicillin G per ml. The occurrence of cellular lysis was controlled by periodic microscopic examination. When at least 90% of the cells had lysed, the survivors were harvested by centrifugation, washed, and plated on agar containing the aromatic compound (2.5 mM) and a growth-limiting concentration of succinate (0.5 mM). Small colonies were purified by restreaking on succinate-agar plates and screened for nutritional defects by patching them on agar plates containing different aromatic substrates.

Thermal inactivation experiments. Samples of cell-free extract (0.25 ml, containing 1 mg of protein) were placed in small test tubes which had been preincubated at the desired temperature. Rapid temperature equilibrium was assured by allowing the extract to flow down the side of the test tube to the bottom in a thin stream. The tube was then gently agitated for 30 sec. Immediately after the addition of the extract, tubes were capped with rubber stoppers. The heat treatment was terminated by plunging the samples into an ice-water bath.

RESULTS

Enzymes induced in wild-type A. eutrophus by growth with intermediates of the β-ketoadipate pathway. Wild-type *A. eutrophus* can grow with four intermediates of the β-ketoadipate pathway: *cis,cis*-muconate, (+)-muconolactone, β-ketoadipate, and protocatechuate. All except (+)-muconolactone support reasonably rapid rates of growth (Table 1).

TABLE 1. *Growth rates of Alcaligenes eutrophus 335 under aerobic conditions at 30 C in synthetic media furnished with several different substrates as sole sources of carbon and energy*[a]

Substrates	Generation time (min)
Succinate	60
Fructose	85
Protocatechuate	70
cis,cis-Muconate	110
(+)-Muconolactone	390
β-Ketoadipate	95

[a] Initial substrate concentration was 2.5 mM in a mineral base medium.

The specific activities of enzymes of the β-ketoadipate pathway in uninduced (succinate-grown) cells and in cells grown with each of these intermediates are compared in Tables 2 and 3. In cells grown with β-ketoadipate (Table 2), the specific activity of β-ketoadipate succinyl-coenzyme A (CoA) transferase is increased about 50-fold over its level in succinate-grown cells and reaches approximately 70% of the level characteristic of benzoate-grown cells. This enzyme accordingly appears to be either substrate-induced by β-ketoadipate or product-induced by β-ketoadipyl-CoA. Two other enzymes, muconolactone isomerase and β-ketoadipate enol-lactone hydrolase, are slightly induced by growth with β-ketoadipate, but their levels are insignificant relative to those in benzoate-grown cells.

Cells grown with *cis,cis*-muconate (Table 2) do not contain a significant level of catechol-1,2-oxygenase (EC 1.13.1.1, catechol:oxygen 1,2-oxido-reductase). Hence, this enzyme is not product-induced by *cis,cis*-muconate, as it is in *Pseudomonas* (12) and in *Acinetobacter* (6).

TABLE 2. *Specific activities[a] of certain enzymes of the β-ketoadipate pathway in extracts of Alcaligenes eutrophus grown at the expense of succinate, benzoate, and certain intermediates in benzoate metabolism[b]*

Enzymes	Growth substrates				
	5 mM Succinate	5 mM *cis,cis*-Muconate	5 mM (+)-Muconolactone	5 mM β-Ketoadipate	5 mM Benzoate
Catechol-1,2-oxygenase	<0.0001	0.0001	<0.0001	0.0001	0.19
Muconate lactonizing enzyme	<0.0001	0.61	0.02	0.0002	0.65
Muconolactone isomerase	0.006	0.64	0.16	0.041	2.68
β-Ketoadipate enol-lactone hydrolase	0.006	0.32	0.11	0.035	1.30
β-Ketoadipate succinyl-CoA transferase	0.009	0.47	0.27	0.44	0.62

[a] Specific activities are expressed at units per milligram of protein.

[b] Cells were grown exponentially at the expense of each substrate for at least five generations before harvesting.

Furthermore, the catechol-1,2-oxygenase of *A. eutrophus* is not present in phenol-grown cells, even though catechol is an intermediate in phenol oxidation (10). Hence it cannot be induced by its substrate. The inducer must therefore be benzoate, or an intermediate in the conversion of benzoate to catechol.

Although the specific activity of muconate lactonizing enzyme [EC 5.5.1.1, 4-carboxymethyl-4-hydroxyisocrotonolactone lyase (decyclizing)] in *cis,cis*-muconate-grown cells is as high as that in benzoate-grown cells, the specific activities of muconolactone isomerase and β-ketoadipate enol-lactone hydrolase are much lower (approximately 25% of the values in benzoate-grown cells). This shows that the synthesis of muconate lactonizing enzyme is noncoordinate with the synthesis of muconolactone isomerase and β-ketoadipate enol-lactone hydrolase. Furthermore, it suggests that an early metabolite of the catechol branch, formed prior to *cis,cis*-muconate, exerts an inductive effect on the synthesis of muconolactone isomerase and β-ketoadipate enol-lactone hydrolase but not on that of muconate lactonizing enzyme.

All the enzymes assayed in extracts of cells grown with (+)-muconolactone showed low specific activities, relative to those characteristic of the same enzymes in cells grown with either benzoate or *cis,cis*-muconate (Table 2). The low activities are correlated with an exceptionally low growth rate of *A. eutrophus* on (+)-muconolactone (Table 1). There is significant induction of β-ketoadipate succinyl-CoA transferase, β-ketoadipate enol-lactone hydrolase, and muconolactone isomerase and a very slight induction of muconate lactonizing enzyme. Of these enzymes, only the first is significantly induced in cells grown with β-ketoadipate; its induction in muconolactone-grown cells can be reasonably attributed to the formation of β-ketoadipate during the dissimilation of muconolactone. Since the conversion of *cis,cis*-muconate to (+)-muconolactone is freely reversible (13), *cis,cis*-muconate could be produced within the cell when *A. eutrophus* is grown with (+)-muconolactone. The inductions of muconolactone isomerase and β-ketoadipate enol-lactone hydrolase, as well as the very weak induction of muconate lactonizing enzyme in muconolactone-grown cells, therefore do not necessarily prove that muconolactone itself can act as an inducer. All three activities could be induced by endogenously formed *cis,cis*-muconate. Induction of muconate lactonizing enzyme and muconolactone isomerase by the enol-lactone of β-ketoadipic acid can be excluded, since this intermediate is also formed from *p*-hydroxybenzoate. We have previously shown (10) that neither of these enzymes is significantly induced in cells grown with *p*-hydroxybenzoate.

As shown in Table 3, the induction of *p*-hydroxybenzoate hydroxylase is negligible in cells grown with protocatechuate. This enzyme must therefore be specifically induced by its substrate, *p*-hydroxybenzoate.

Responses to induction of mutants unable to synthesize p-hydroxybenzoate hydroxylase and protocatechuate oxygenase. Mutant pOHB.1, which has lost the ability to synthesize *p*-hydroxybenzoate hydroxylase, does not synthesize any other enzymes of the pathway when grown in the presence of *p*-hydroxybenzoate with succinate as a carbon source (Table 4). Grown under comparable conditions, the wild type synthesized high levels of all the enzymes of the protocatechuate branch; hence, the failure of this mutant to respond to *p*-hydroxybenzoate as an inducer is not attributable to catabolite repression by succinate. Grown with protocatechuate, the enzymatic constitution of this mutant is indistinguishable from that of the wild type. These experiments show that *p*-hydroxybenzoate hydroxylase is the only enzyme of the protocatechuate branch induced by *p*-hydroxybenzoate.

Several independently isolated mutants which had lost the ability to synthesize protocatechuate oxygenase (EC 1.13.1.3, protocatechuate:

oxygen 3-4 oxidoreductase) were examined. When grown in the presence of protocatechuate with succinate as a carbon source, mutant PCA.5 contained high levels of β-carboxymuconate lactonizing enzyme, γ-carboxymuconolactone decarboxylase, and β-ketoadipate enol-lactone hydrolase; however, β-ketoadipate succinyl-CoA transferase was not induced (Table 5).

TABLE 3. *Specific activities[a] of certain enzymes of the β-ketoadipate pathway in extracts of Alcaligenes eutrophus grown with succinate, protocatechuate, and p-hydroxybenzoate[b]*

Enzymes	Growth substrates		
	5 mM Succinate	5 mM Protocatechuate	5 mM *p*-Hydroxybenzoate
p-Hydroxybenzoate hydroxylase	<0.0001	0.005	0.22
Protocatechuate oxygenase	0.001	0.29	0.84
β-Carboxymuconate lactonizing enzyme	0.003	0.90	2.31
γ-Carboxymuconolactone decarboxylase	0.021	1.36	2.46
β-Ketoadipate enol-lactone hydrolase	0.006	0.62	1.53
β-Ketoadipate succinyl-CoA transferase	0.009	0.65	0.66

[a] Expressed as units per milligram of protein.
[b] Cells were grown exponentially at the expense of each substrate for at least five generations before harvesting.

Two other mutants unable to synthesize protocatechuate oxygenase showed a somewhat different response when grown with succinate in the presence of protocatechuate. Both contained high levels of γ-carboxymuconolactone decarboxylase and β-ketoadipate enol-lactone hydrolase, but β-carboxymuconate lactonizing enzyme was barely detectable in mutant PCA.4 and present at a comparatively low level in mutant PCA.1 (Table 5). Both mutants produced revertants when plated on a medium that contained *p*-hydroxybenzoate as sole carbon source, and the revertants were indistinguishable from wild type with respect to their inductive responses. Strains PCA.1 and PCA.4 accordingly appear to be polarity mutants.

Coordinate synthesis of enzymes in the protocatechuate branch of the pathway. Since protocatechuate cannot be metabolized by mutants lacking protocatechuate oxygenase, it was possible to study the effect of protocatechuate concentration on the synthesis of β-carboxymuconate lactonizing enzyme, γ-carboxymuconolactone decarboxylase, and β-ketoadipate enol-lactone hydrolase in such mutants. The results of an experiment with mutant PCA.5 are shown in Fig. 2. The affinity of induction is exceedingly high, half-maximal levels of activity for all three en-

TABLE 4. *Specific activities[a] of certain enzymes of the β-ketoadipate pathway in extracts of the wild type and of a mutant (POHB.1) unable to synthesize p-hydroxybenzoate hydroxylase*

Enzymes	Wild-type *A. eutrophus* 335		Mutant POHB.1	
	10 mM Succinate-2 mM *p*-hydroxybenzoate	10 mM Succinate-2 mM protocatechuate	10 mM Succinate-2 mM-*p*-hydroxybenzoate	10 mM Succinate-2 mM protocatechuate
p-Hydroxybenzoate hydroxylase	0.039	0.005	0.003	0.002
Protocatechuate oxygenase	0.13	0.17	0.003	0.12
β-Carboxymuconate lactonizing enzyme	0.38	0.31	0.004	0.30
β-Ketoadipate enol-lactone hydrolase	0.29	0.42	0.022	0.37
β-Ketoadipate succinyl-CoA transferase	0.38	0.39	0.017	0.34

[a] Expressed as units per milligram of protein.

TABLE 5. *Specific activities[a] of certain enzymes of the β-ketoadipate pathway in extracts of three mutants unable to synthesize protocatechuate oxygenase*

Enzymes	Mutant PCA.5		Mutant PCA.4	Mutant PCA.1
	10 mM Succinate	10 mM Succinate-2 mM protocatechuate	10 mM Succinate-2 mM protocatechuate	10 mM Succinate-2 mM protocatechuate
Protocatechuate oxygenase	<0.0003	<0.0003	<0.0003	<0.0003
β-Carboxymuconate lactonizing enzyme	0.002	1.96	0.003	0.163
γ-Carboxymuconolactone decarboxylase	0.02	1.99	1.36	1.53
β-Ketoadipate enol-lactone hydrolase	0.01	1.04	1.43	1.29
β-Ketoadipate succinyl-CoA transferase	0.02	0.01	0.03	0.02

[a] Expressed as units per milligram of protein.

zymes being elicited by growth in the presence of 1.4×10^{-6} M protocatechuate. As Fig. 2 also shows, the affinities of induction of the three enzymes are indistinguishable. Taken in conjunction with the polarity effects observed in strains PCA.1 and PCA.4, this suggests that the enzymes in question may be controlled through a complex operon.

Response to induction of mutants blocked at early steps in the catechol branch. Several mutants unable to grow with benzoate were isolated. With one exception, they had lost the ability to synthesize catechol-1,2-oxygenase, as shown by the fact that they grew normally with *cis,cis*-muconate and excreted large amounts of catechol when grown in the presence of benzoate. Since catechol is highly toxic for *A. eutrophus*, it proved difficult to grow these mutants in the presence of benzoate with succinate as a carbon source. The accumulation of catechol could, however, be prevented by the use of phenol as a carbon source. As we showed previously (10), catechol is an intermediate in the dissimilation of phenol and is metabolized through the *meta* cleavage pathway, as a result of the fact that phenol induces catechol-2,3-oxygenase and later enzymes of this pathway. Consequently, when mutants unable to synthesize catechol-1,2-oxygenase are exposed to a mixture of phenol and benzoate, the catechol formed from benzoate is destroyed through the activity of catechol-2,3-oxygenase, and normal growth occurs.

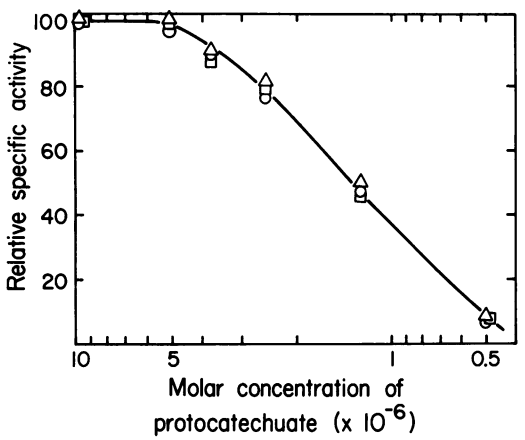

FIG. 2. *Relative specific activities of certain enzymes in extracts of mutant PCA.5, after growth for three generations in the presence of different concentrations of protocatechuate. The specific activity of each enzyme is expressed relative to its specific activity in an extract of cells grown in the presence of 10^{-5} M protocatechuate, which elicited maximal induction. These values are plotted as a function of the concentration of protocatechuate to which the cells were exposed during induction. Carboxymuconate lactonizing enzyme* (△), *carboxymuconolactone decarboxylase* (○), *and β-ketoadipate enol-lactone hydrolase* (□).

The enzymatic constitutions of two catechol-1,2-oxygenaseless mutants grown with phenol in the presence of benzoate are shown in Table 6. Two enzymes operative below the mutationally blocked step, muconolactone isomerase and β-ketoadipate enol-lactone hydrolase, are induced. This cannot be attributed to leakiness of the mutants, since muconate lactonizing enzyme is not significantly induced. Neither muconolactone isomerase nor β-ketoadipate enol-lactone hydrolase is induced when mutant B.5 is grown with phenol alone (Table 6). Hence muconolactone isomerase and β-ketoadipate enol-lactone hydrolase must be induced by benzoate or by an intermediate in its conversion to catechol.

One mutant selected for inability to grow with benzoate, strain B.9, also grew very poorly with *cis,cis*-muconate and produced a substance which absorbed strongly at 260 nm when exposed to benzoate. It was initially interpreted as having an impaired capacity to synthesize muconate lactonizing enzyme. However, a revertant se-

TABLE 6. *Specific activities*[a] *of certain enzymes of the β-ketoadipate pathway in extracts of two mutants blocked at different steps in the catechol branch*[b]

Enzymes	Mutant B.9 CCMR			Mutant B.5		
	5 mM Fructose	4 mM Fructose-1 mM benzoate	3 mM *cis,cis*-Muconate	4 mM Phenol	3 mM Phenol 1 mM benzoate	3 mM *cis,cis*-Muconate
Catechol-1,2-oxygenase	<0.0001	0.17	0.0001	0.0003	0.0003	0.0001
Muconate lactonizing enzyme	<0.0001	0.009	0.60	<0.0001	0.012	0.56
Muconolactone isomerase	0.018	0.75	0.54	0.022	0.93	0.45
β-Ketoadipate enol-lactone hydrolase	0.027	0.36	0.34	0.025	0.45	0.25
β-Ketoadipate succinyl-CoA transferase	0.01	0.02	0.36	0.01	0.03	0.53

[a] Expressed as units per milligram of protein.

[b] Mutant B.9 CCMR is unable to convert 1,2-dihydro-1,2-dihydroxybenzoate to catechol; mutant B.5 is unable to synthesize catechol-1,2-oxygenase.

lected for its ability to grow well with *cis,cis*-muconate (strain B.9 CCMR) still failed to grow with benzoate and still accumulated a substance absorbing at 260 nm when exposed to benzoate. This compound is 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid, a hitherto unknown intermediate between benzoate and catechol for which the trivial name 1,2-dihydro-1,2-dihydroxybenzoic acid has been proposed (Reiner and Hegeman, *in press*).

We have studied the inductive responses of the partial revertant, strain B.9 CCMR, in which the metabolism of muconate is normal. After growth in the presence of benzoate, this strain contains high levels of catechol-1,2-oxygenase, muconolactone isomerase, and β-ketoadipate enol-lactone hydrolase. These three enzymes are therefore all induced either by benzoate itself or by the intermediate compound, 1,2-dihydro-1,2-dihydroxybenzoate, which accumulates and is excreted by this mutant.

Conversion of wild-type A. eutrophus to a phenocopy of a catechol-1,2-oxygenaseless mutant. Although *A. eutrophus* 335 grows well at 37 C with most substrates, it cannot do so with benzoate; cells exposed to benzoate at 37 C accumulate and excrete large amounts of catechol. The synthesis of catechol-1,2-oxygenase is accordingly temperature-sensitive in the wild type. This peculiarity makes it possible to study the inductive properties of benzoate in the wild type under conditions in which the steps of the pathway below catechol are inoperative for physiological reasons. As shown in Table 7, the enzymatic constitution of the wild type induced by benzoate while growing at 37 C with succinate and phenol as carbon sources is indistinguishable from that of a catechol-1,2-oxygenaseless mutant (*see* Table 6).

Coordinate control of enzymes in the catechol branch. In Table 8, the relative specific activities of enzymes of the catechol branch in wild type and mutant strains grown under widely different conditions of induction are compared (data from the experiments presented in Tables 2, 6, and 7). For each enzyme, specific activities are expressed relative to that in cells of the wild type grown with benzoate, assigned an arbitrary value of 100. A close parallelism between the activities of muconolactone isomerase and β-ketoadipate enol-lactone hydrolase is maintained under all conditions, which indicates that these two enzymes are subject to coordinate regulation. None of the other enzymes of the catechol branch seems to be coordinately controlled.

Enzymology of the terminal common step-reactions. The two branches of the β-ketoadipate pathway converage metabolically with the forma-

TABLE 7. *Specific activities[a] of certain enzymes of the β-ketoadipate pathway in extracts of wild-type Alcaligenes eutrophus 335 grown at 37 C under the conditions shown*

Enzymes	Growth substrates	
	5 mM Succinate–1 mM phenol	5 mM Succinate–1 mM phenol–1 mM benzoate
Catechol-1,2-oxygenase	<0.0005	<0.0005
Muconate lactonizing enzyme	<0.0001	0.0002
Muconolactone isomerase	0.012	1.45
β-Ketoadipate enol-lactone hydrolase	0.017	0.77
β-Ketoadipate succinyl-CoA transferase	0.01	0.05

[a] Expressed as units per milligram of protein.

TABLE 8. *Relative specific activities of certain enzymes of the β-ketoadipate pathway in extracts of wild-type Alcaligenes eutrophus and of two mutants grown under a variety of conditions[a]*

Enzymes	Strain					
	Wild type		B.5	B.9 CCMR	Wild type	
	4 mM Benzoate	5 mM Succinate[b]–1 mM benzoate–1 mM phenol	3 mM Phenol–1 mM benzoate	4 mM Fructose–1 mM benzoate	4 mM *cis,cis*-Muconate	4 mM (+)-Muconolactone
Catechol-1,2-oxygenase	100			90	0.5	<0.5
Muconate lactonizing enzyme	100	0.3	1.8	1.4	94	3
Muconolactone isomerase	100	54.0	35	28	24	6
β-Ketoadipate enol-lactone hydrolase	100	59.0	35	28	25	8
β-Ketoadipate succinyl-CoA transferase	100	8.0	4.4	2.4	76	44

[a] The specific activity of each enzyme in benzoate-grown cells of the wild type is assigned a value of 100; the specific activities in cells grown under other conditions are expressed relative to these values.

[b] Grown at 37 C.

tion of the enol-lactone of β-ketoadipate, which is converted to β-ketoadipyl-CoA through the successive action of the two enzymes, β-ketoadipate enol-lactone hydrolase and β-ketoadipate succinyl-CoA transferase. In *A. eutrophus*, the second of these enzymes, β-ketoadipate succinyl-COA transferase, appears to be induced either by its product or by its substrate. However, the data already presented point to the existence of two different enzymes with β-ketoadipate enol-lactone hydrolase function, separately inducible by intermediates in the catechol and protocatechuate branches of the pathway. Further evidence for the existence of two inducible enzymes with β-ketoadipate enol-lactone hydrolase function in *A. eutrophus* was obtained by a study of the kinetics of thermal inactivation of this activity in extracts prepared from cells subjected to different conditions of induction. As a control, the kinetics of thermal inactivation of β-ketoadipate succinyl-CoA transferase were determined on the same extracts.

The results of these experiments are shown in Fig. 3 and 4. It is evident that the rate of thermal inactivation of β-ketoadipate succinyl-CoA transferase at 49 C is unaffected by the

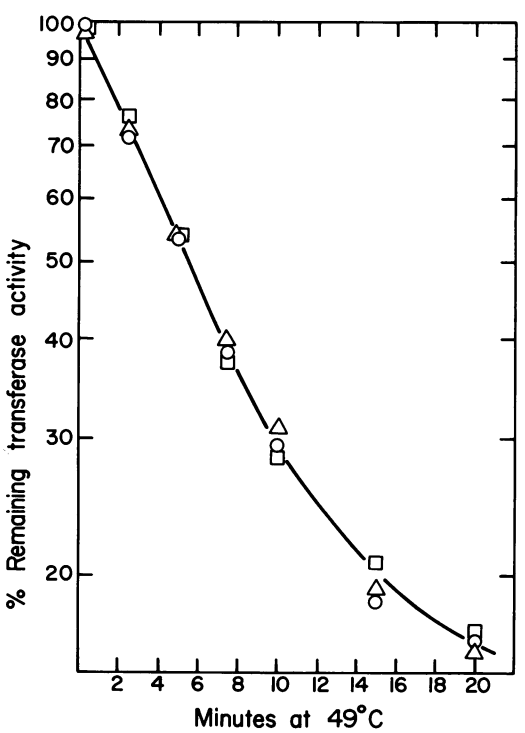

FIG. 3. *Kinetics of thermal inactivation of β-ketoadipate succinyl-CoA transferase activity at 49 C in cell-free extracts of Alcaligenes eutrophus 335 grown with benzoate* (○), *p-hydroxybenzoate* (△), *and adipate* (□).

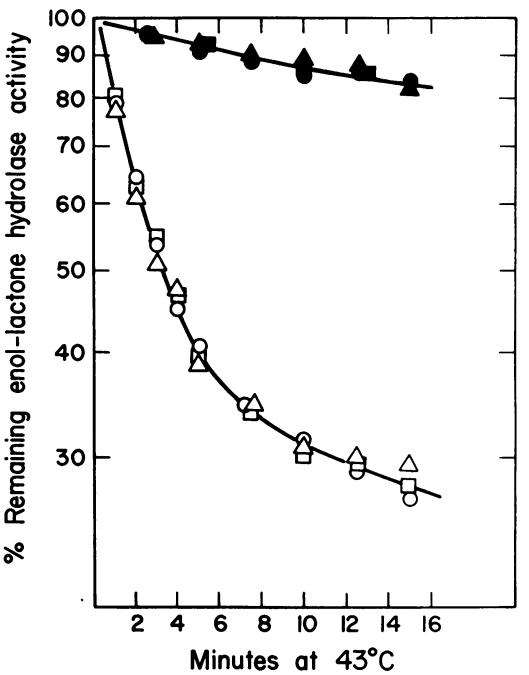

FIG. 4. *Kinetics of thermal inactivation of β-ketoadipate enol-lactone hydrolase activity at 43 C in cell-free extracts of Alcaligenes eutrophus. Wild-type strain (335) grown with p-hydroxybenzoate* (■), *protocatechuate* (●), *benzoate* (□), *and cis,cis-muconate* (○). *Two mutants in which activity was induced under conditions of gratuity: mutant PCA.5 grown in the presence of protocatechuate* (▲); *and mutant B.5 grown in the presence of benzoate* (△).

nature of the growth substrate. This suggests (but does not prove) that *A. eutrophus* can synthesize only one enzyme with β-ketoadipate succinyl-CoA transferase function. However, the rate of thermal inactivation of β-ketoadipate enol-lactone hydrolase is markedly affected by the nature of the substrate with which the cells have been grown. The enzyme induced by growth with either benzoate or *cis,cis*-muconate is rapidly destroyed at 43 C, although a minor fraction of the activity is relatively thermostable. These experiments confirm the existence in *A. eutrophus* of two different enzymes with β-ketoadipate enol-lactone hydrolase function. One, which we shall term β-ketoadipate enol-lactone hydrolase I, is induced by protocatechuate and is operative in the protocatechuate branch of the pathway. The other, which we shall term β-ketoadipate enol-lactone hydrolase II, is operative in the catechol branch of the pathway; its induction is complex.

DISCUSSION

The system of regulation governing the syn-

thesis of the enzymes of the β-ketoadipate pathway by *A. eutrophus*, insofar as it can be inferred from the experiments described here, is shown in Fig. 5. The control of the enzymes operative in the protocatechuate branch of the pathway is relatively simple and has been established without ambiguity by our experiments. The first enzyme, *p*-hydroxybenzoate hydroxylase, is induced only by its substrate, *p*-hydroxybenzoate. The other four enzymes of the branch (protocatechuate oxygenase, β-carboxymuconate lactonizing enzyme, γ-carboxymuconolactone decarboxylase, and β-ketoadipate enol-lactone hydrolase I) are all induced by protocatechuate. As shown by the inductive response of a mutant unable to synthesize protocatechuate oxygenase, the synthesis of the three remaining enzymes is strictly coordinate. Furthermore, some of the mutants which lack protocatechuate oxygenase show polarity effects with respect to the synthesis of the other three enzymes. Hence, it is very probable that all four enzymes are controlled by a single complex operon.

The control of the synthesis of enzymes operative in the catechol branch is much more complex, and most of the inducers cannot yet be identified with certainty. Two enzymes of this branch, muconolactone isomerase and β-ketoadipate enol-lactone hydrolase II, are subject to coordinate control and exhibit a unique mode of regulation, since their synthesis can be elicited by two different and sterically unrelated metabolite inducers. One of these inducers is identifiable as either benzoate or 1,2-dihydro-1,2-dihydroxy benzoate; the other as either *cis,cis*-muconate or (+)-muconolactone. The dual regulatory control of these two enzymes is shown by the fact that they are induced in cells grown with muconate or muconolactone, and also in cells grown in the presence of benzoate but incapable of metabolizing this compound beyond the level of catechol. From the levels of activity elicited under these different conditions of induction, it is evident that the earlier of the two metabolite-inducers (namely, benzoate or its 1,2-dihydro-1,2-dihydroxy derivative) is the more effective one.

Muconate lactonizing enzyme is probably substrate-induced by *cis,cis*-muconate, since it is present at high levels in muconate-grown cells but at very low levels in cells grown with its product, (+)-muconolactone. Catechol-1,2-oxygenase is not induced either by its substrate or its product; the inducer must be benzoate or its 1,2-dihydro-1,2-dihydroxy derivative.

Even though some ambiguities remain, the data now available show that the regulation of the β-ketoadipate pathway in *A. eutrophus* is different in many respects from the regulation of

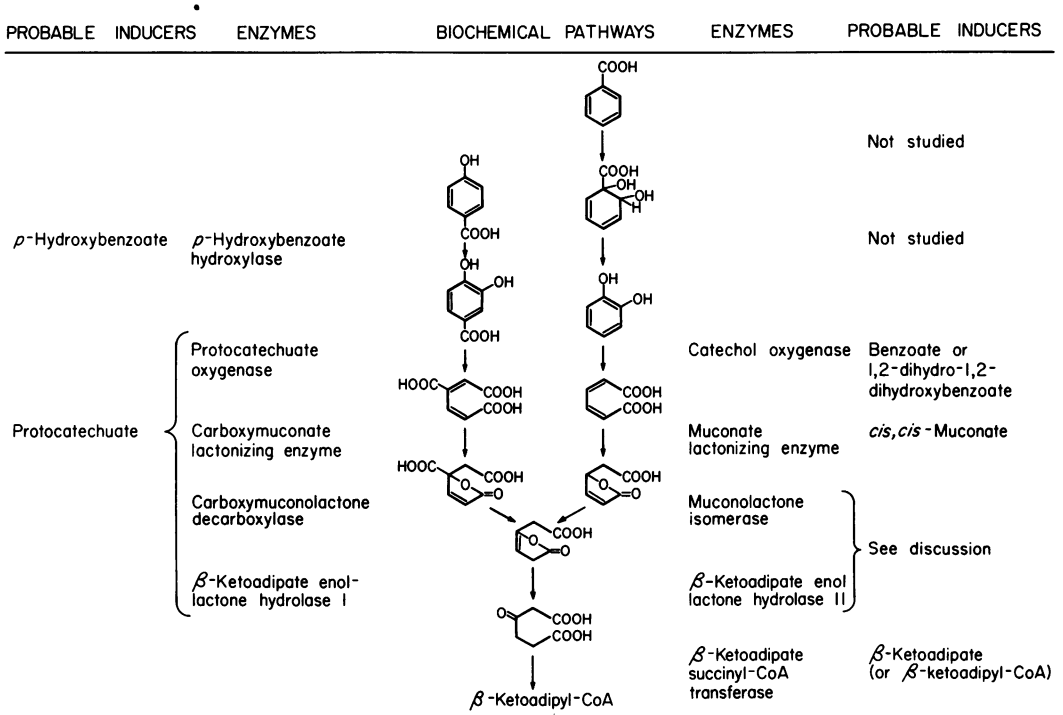

FIG. 5. *System of regulation governing the synthesis of the enzymes operative in the β-ketoadipate pathway in Alcaligenes eutrophus.*

the same pathway in the genera *Pseudomonas* and *Acinetobacter*. The regulatory systems characteristic of each genus are compared schematically in Fig. 6.

Three of the nonaromatic intermediates of this pathway are chemically very unstable: β-carboxy-*cis*,*cis*-muconate and γ-carboxymuconolactone, the last intermediates specific to the protocatechuate branch; and the enol-lactone of β-ketoadipic acid, the first intermediate common to both branches of the pathway. None of these intermediates functions as an inducer in any of the three genera; as a result, the regulation of the three enzymes for which they serve as substrates—β-carboxymuconate lactonizing enzyme, γ-carboxymuconolactone decarboxylase, and β-ketoadipate enol-lactone hydrolase—is in each case somewhat complex. In the genus *Pseudomonas*, these three enzymes are induced by a late metabolite common to both branches of the pathway (β-ketoadipate or β-ketoadipyl-CoA). In *Acinetobacter* and in *Alcaligenes*, the three enzymes are coordinately induced by an early intermediate of the protocatechuate branch, protocatechuate; these organisms synthesize a second enzyme with β-ketoadipate enol-lactone hydrolase function, subject to separate inductive control by an intermediate of the catechol branch. The *Pseudomonas* control system leads to gratuitous induction of two enzymes operative in the catechol branch (carboxymuconate lactonizing enzyme and carboxymuconolactone decarboxylase) when cells are grown with a substrate metabolized through the catechol branch. The *Achromobacter* and *Acinetobacter* control systems necessitate two separate structural genes and regulatory systems governing the synthesis of β-ketoadipate enol-lactone hydrolase. In principle, all these complexities could be circumvented by the interposition of a sequential induction following the point of metabolic convergence. It is curious that this seemingly simple solution of the regulatory problem posed by metabolic convergence has not been employed in any of the bacterial groups so far examined.

The control of the last enzyme of the pathway, β-ketoadipate succinyl-CoA transferase, likewise differs in the three bacterial groups. The simplest control mechanism occurs in *Alcaligenes*, which synthesizes a single enzyme with this function, induced either by its substrate or by its product, independently of all other enzymes of the

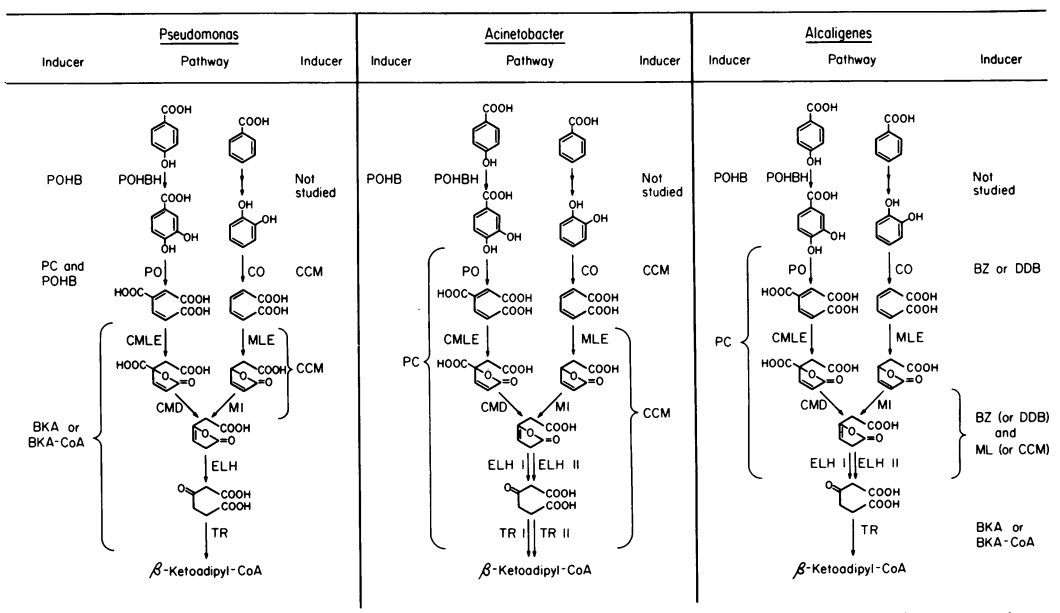

FIG. 6. *Comparison of the mechanisms of regulation that govern synthesis of the enzymes of the β-ketoadipate pathway in three different genera of aerobic, gram-negative bacteria. Data for Pseudomonas taken from Ornston (12) and Kemp and Hegeman (11); data for Acinetobacter taken from Cánovas and Stanier (6); data for Alcaligenes from the present study. Brackets denote groups of enzymes whose synthesis is subject to coordinate regulation. Abbreviations for enzymes: POHBH, p-hydroxybenzoate hydroxylase; PO, protocatechuate oxygenase; CMLE, β-carboxymuconate lactonizing enzyme; CMD, carboxymuconolactone decarboxylase; ELH, β-ketoadipate enol-lactone hydrolase; TR, β-ketoadipate succinyl-CoA transferase; CO, catechol oxygenase; MLE, muconate lactonizing enzyme; MI, muconolactone isomerase. Abbreviations for inducer-metabolites: POHB, p-hydroxybenzoate; PC, protocatechuate; BKA, β-ketoadipate; BZ, benzoate; DDB, 1,2-dihydro-1,2-dihydroxybenzoate; CCM, cis,cis-muconate; ML, (+)-muconolactone.*

pathway. *Pseudomonas* species also synthesize a single β-ketoadipate succinyl-CoA transferase which is elicited by the same inducer(s) (11, 12). However, its synthesis is coordinate with those of carboxymuconate lactonizing enzyme, carboxymuconolactone decarboxylase, and enol-lactone hydrolase. Genetic data (11, 15) strongly suggest that the four enzymes are all controlled by a single operon. The most complex system for the regulation of β-ketoadipate succinyl-CoA transferase activity occurs in *Acinetobacter*, which possesses two enzymes with this function. One is synthesized coordinately with enzymes of the catechol branch and is induced by *cis,cis*-muconate, and the other is synthesized coordinately with enzymes of the protocatechuate branch and is induced by protocatechuate (6).

As a consequence of these differences with respect to regulation, the two branches of the pathway converge enzymologically at different points in each genus. In *Pseudomonas*, metabolic and enzymological convergence occur at the same point (viz., with formation of the β-ketoadipate enol-lactone). In *Alcaligenes*, enzymological convergence occurs one step later (viz., with the formation of β-ketoadipate). In *Acinetobacter*, the point of enzymological convergence is still unknown, since the two branches of the pathway remain enzymologically separate up to and including the formation of β-ketoadipyl-CoA.

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