JOURNAL OF BACTERIOLOGY, Oct. 1995, p. 5826–5833 Vol. 177, No. 20
0021-9193/95/$04.00+0

Acetate Utilization Is Inhibited by Benzoate in *Alcaligenes eutrophus*: Evidence for Transcriptional Control of the Expression of *acoE* Coding for Acetyl Coenzyme A Synthetase

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Received 27 March 1995/Accepted 4 August 1995

During batch growth of *Alcaligenes eutrophus* on benzoate-acetate mixtures, benzoate was the preferred substrate, with acetate consumption being delayed until the rate of benzoate consumption had diminished. This effect was attributed to a transcriptional control of the synthesis of acetyl coenzyme A (acetyl-CoA) synthetase, an enzyme necessary for the entry of acetate into the central metabolic pathways, rather than to a biochemical modulation of the activity of this enzyme. Analysis of a 2.4-kb mRNA transcript hybridizing with the *A. eutrophus acoE* gene confirmed this repression effect. In a benzoate-limited chemostat culture, derepression was observed, with no increase in the level of expression following an acetate pulse. Benzoate itself was not the signal triggering the repression of acetyl-CoA synthetase. This role was played by catechol, which transiently accumulated in the medium when high specific rates of benzoate consumption were reached. The lack of rapid inactivation of the functional acetyl-CoA synthetase after synthesis has been stopped enables *A. eutrophus* to retain the capacity to metabolize acetate for prolonged periods while conserving minimal protein expenditure.

Many soil bacteria are capable of mineralizing aromatic compounds. Among these, *Alcaligenes eutrophus* seems to be an organism of choice with regard to its versatility as regards the large number of substrates metabolized (7), the capacity to use nitrate as an alternative electron acceptor (5), and its chromosome-encoded pathways for the degradation of aromatic compounds (20). In addition, plasmids allow this bacterium to grow chemolithotrophically (6, 15), to be resistant to high heavy-metal concentrations (10), and to extend the range of degraded substrates to pollutants such as 2,4-dichlorophenoxyacetate (36), chlorobenzenes (11), methylaromatics (33), or polychlorinated biphenyls (3, 41).

In natural environments, various alternative substrates are usually present although often at concentrations sufficiently low that microbial growth can be considered to be carbon limited. Under such conditions, simultaneous consumption of all metabolizable carbon sources is to be expected. However, periods of carbon excess will occur, and while some microorganisms can simultaneously consume many carbon substrates (2), others exert some degree of preference for specific substrates, generally metabolizing those supporting most efficient growth. The repression of catabolism of less favorable substrates by other carbon sources (often referred to as catabolite repression) has been extensively described for enteric bacteria (4) and a few other genera such as *Bacillus* (9) and *Pseudomonas* (23). The "catabolite repression" effect in the last two organisms, closely related to *A. eutrophus*, is not understood yet but has been shown to be cyclic AMP (cAMP) independent (18, 23, 24, 32). Possible interactions between the consumption of aromatic compounds and other carbon substrates have received little attention despite the importance that such metabolic regulation phenomena may have in explaining the recalcitrant nature of such pollutants within conventional treatment processes.

The simple aromatic compound benzoate is metabolized by *A. eutrophus* via the *ortho* (also named β-ketoadipate) pathway (Fig. 1), which has received far less study than the plasmid-encoded *meta* pathways of aromatic compound degradation. The highly complex biochemical regulation of the β-ketoadipate pathway was partly elucidated for *A. eutrophus* 335 by Johnson and Stanier (21). Benzoate and *cis*,*cis*-muconate appeared to be key intermediates in the induction of the enzymes of the pathway (21, 42), but despite those early reports there remain gaps in the knowledge of the regulation, and to date only two genes of the pathway (*catD* and *pcaD*, coding for the two lactone hydrolases of *A. eutrophus*) have been cloned and studied (39).

The β-ketoadipate pathway results in the production of succinate and acetyl coenzyme A (acetyl-CoA), which are then metabolized by the central metabolic pathways. This interface is a logical site for regulatory control, and indeed succinate has been clearly demonstrated to have a profound inhibitory effect on the synthesis of the enzymes of the β-ketoadipate pathway of *Pseudomonas putida* (30). Little additional information was reported until Zylstra et al. (45) demonstrated that the expression of the two *P. cepacia* protocatechuate-3,4-dioxygenase genes was constitutive but subject to catabolite repression by succinate. Catabolite repression of the *pcd* (protocatechuate-3,4-dioxygenase) gene from *Bradyrhizobium japonicum* USDA 110 was also observed in the presence of glucose in complemented *pcd* mutants of *P. cepacia* (34). Finally, a recent report has shown that the presence of nonlimiting concentrations of succinate in continuous cultures of *P. putida* pWW0 was sufficient to block the expression of the *o*-xylene degradation pathway at the transcriptional level (12).

So far, very little attention has been paid to the role of acetyl-CoA or the direct precursor, acetate, despite the com-

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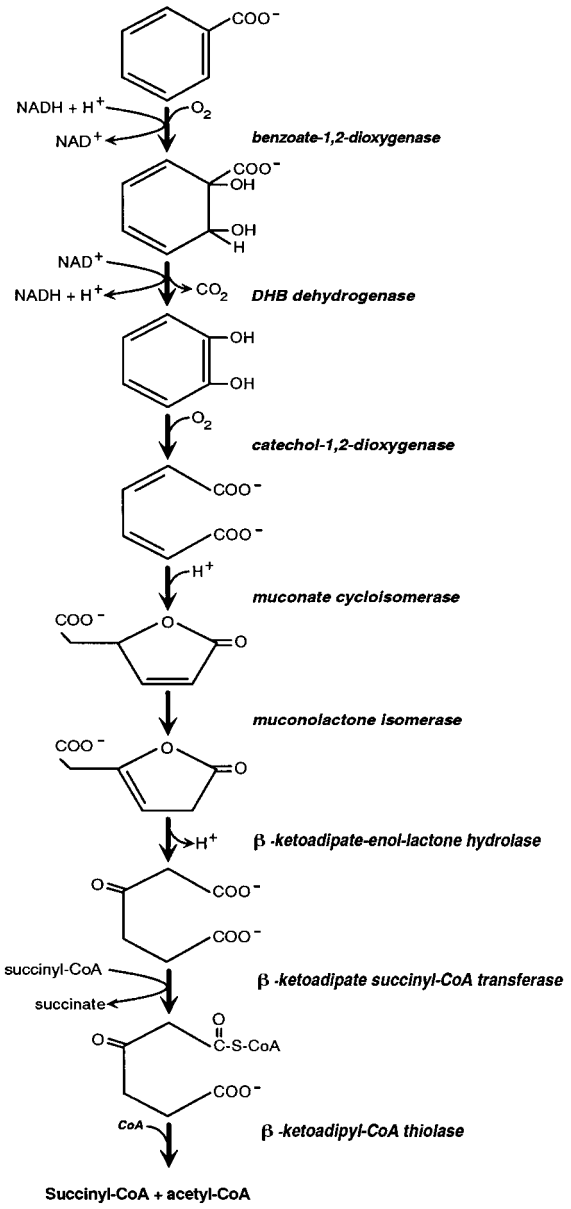

FIG. 1. The β-ketoadipate pathway (also named the *ortho*-cleavage pathway) for the degradation of benzoate in *A. eutrophus* (adapted from references 20 and 37).

mon occurrence of this compound in natural environments and the well-documented repression by acetate of a number of enzymes necessary for sugar assimilation by *P. aeruginosa* (44). Recently, acetate (as well as gluconate and lactate) was also shown to exert a repression effect on the promoters Ps and Pu in *P. putida* pWW0 (18). In *A. eutrophus*, acetate metabolism is initiated by acetyl-CoA formation, involving acetyl-CoA synthetase and not the more commonly reported acetate kinase/phosphotransacetylase mechanism (43). However, the metabolic control regulating competition between acetate and aromatic compounds in *A. eutrophus* remains largely obscure. To our knowledge, the only aromatic compound investigated in this light is phenol, whose biodegradation via the *meta* pathway was shown to be delayed in the presence of acetate. Hughes and Bayly (19) claimed a total repression of phenol hydroxylase and a 50% decrease in the activity of the other enzymes of the *meta* pathway in *A. eutrophus*. Schmidt and Alexander (40) also reported diminished degradation of phenol in *Pseudomonas* strains when grown in the presence of acetate.

In this study, the metabolic phenomena involved during the growth of *A. eutrophus* on benzoate-acetate mixtures has been investigated. The results demonstrate that in contrast to reports concerning phenol, acetate metabolism is repressed during periods of rapid benzoate consumption. Transcript analysis has enabled this phenomenon to be localized at the level of transcription of the *acoE* (acetyl-CoA synthetase) gene.

MATERIALS AND METHODS

Bacterial strains and plasmids. *A. eutrophus* 335 (ATCC 17697) was obtained from LMG, Brussels, Belgium. *A. eutrophus* HF149 (*rpoN*) (17) was kindly provided by Bärbel Friedrich. *A. eutrophus* B9 lacking 1,2-dihydro-1,2-dihydroxybenzoate (DHB) dehydrogenase (37) was kindly provided by George Hegeman. *Escherichia coli* XL1-Blue harboring plasmid pSES38 described by Priefert and Steinbüchel (35) was kindly provided by Horst Priefert. pSES38 is a hybrid plasmid constructed from pBluescript SK$^-$ and harboring a 3.8-kb *Eco*RI-*Sma*I subfragment including the *A. eutrophus acoE* gene for acetyl-CoA synthetase.

Growth of bacteria. The mineral salts medium used for growth of *A. eutrophus* was derived from that described by Johnson and Stanier (20) and contained nitrilotriacetic acid, 200 mg/liter; $FeSO_4 \cdot 7H_2O$, 7 mg/liter; $MgSO_4 \cdot 7H_2O$, 580 mg/liter; $CaCl_2 \cdot 2H_2O$, 67 mg/liter; NaCl, 292 mg/liter; $(NH_4)_2SO_4$, 2 g/liter; $ZnSO_4 \cdot 7H_2O$, 10.95 mg/liter; $MnSO_4 \cdot H_2O$, 1.54 mg/liter; $CuSO_4$, 0.251 mg/liter; $CoCl_2 \cdot 6H_2O$, 0.2 mg/liter; H_3BO_4, 0.114 mg/liter; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 2 mg/liter; and $NiCl_2 \cdot 6H_2O$, 0.04 mg/liter; the pH of the basal salt medium was adjusted to 7.4, and the medium was autoclaved. A stock solution of 1 M potassium phosphate (pH 7.4) was autoclaved separately and added to a final concentration of 40 mM in the medium. Carbon sources were filter sterilized. Unless otherwise stated, sodium benzoate was used at 5 mM and sodium acetate was used at 60 mM. A 1.5-liter bioreactor from Setric, Toulouse, France, was used for the present study. The temperature was maintained constant at 30°C, the pH was maintained at 7.4 with controlled addition of H_3PO_4 (1 M), and the oxygen partial pressure was maintained at 60% saturation with air (i.e., around 0.132 mM oxygen dissolved in the medium under these conditions) via agitation and air flow rate variation. The bioreactor was inoculated with 10% (vol/vol) late-exponential-phase inoculum grown in shake flasks on either benzoate or acetate medium, as indicated in the text below. After inoculation, samples were periodically withdrawn from the bioreactor with sterile syringes. Batch cultures were performed in triplicate. For chemostat study, culture conditions were similar to those of batch culture except that the benzoate concentration in the inflowing medium was fixed at 20 mM. The dilution rate was maintained constant at 0.3 h^{-1}, a value close to maximum growth rates obtained on acetate. Acetate pulse experiments were performed by the sudden addition (at $t = 0$) of 28 ml of a sterile 2.5 M sodium acetate solution to obtain an acetate concentration of 60 mM in the bioreactor.

For the preparation of pSES38, *E. coli* XL1-Blue(pSES38) was grown on Luria-Bertani medium (38) with 12.5 mg of tetracycline per liter and 100 mg of ampicillin per liter.

Measurement of fermentation parameters. Biomass was measured by cell dry weight determination. A biomass formula of $C_4H_{6.9}N_{0.98}O_{1.63}$ (with 5% ash) determined by elemental analysis was used for calculations. The concentrations of benzoate and acetate were analyzed by high-pressure liquid chromatography (HP 1050; Hewlett Packard, Grenoble, France) equiped with an integrator (HP 3396A) and an automatic sampler (SP 8775; Spectra Physics France, Les Ulis, France). Detection was made at 210 nm with a variable-wavelength detector (Hewlett Packard, HP series 1050). The separation was performed with an Aminex HPX-87H (Bio-Rad Chemical Division, Richmond, Calif.) column (300 by 7.8 mm), and the operating conditions were as follows: temperature, 65°C; mobile phase, 5 mM H_2SO_4–7% (vol/vol) CH_3CN; flow rate, 0.8 ml/min. Detection limits were 10 μM for benzoate and 100 μM for acetate.

Determination of enzyme activities. Approximately 50 to 100 mg (wet weight) of freshly harvested cells was washed twice in 100 mM Tris.HCl (pH 7.5) at 4°C and resuspended into 10 ml of Tris.carballylate buffer (pH 7.8) (9 mM tricarballylic acid, 35 mM Tris.HCl, 5 mM $MgCl_2$, 20% [vol/vol] glycerol). The cells were disrupted by sonication, and the resulting crude extracts were centrifuged (15,000 × *g* for 20 min at 4°C) to obtain soluble extracts, which were used to assay enzyme activities. Acetyl-CoA synthetase activity (acetate:CoA ligase [AMP forming]; EC 6.2.1.1) and acyl-CoA synthetase activity (propionate:CoA or butyrate:CoA ligase [AMP forming]) were determined by the enzyme assay procedure of Oberlies et al. (29), in which the formation of AMP from ATP is monitored by coupling the reaction to the oxidation of NADH via adenylate kinase, pyruvate kinase, and lactate dehydrogenase. Blanks without CoA and ATP were prepared for each extract. Isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2) activities were determined at pH 7.5 by the procedures described by Maloy et al. (25). Enzymes of the β-ketoadipate pathway were

assayed by published methods: catechol-1,2-dioxygenase (EC 1.13.1.1; catechol: oxygen 1,2-oxidoreductase [28]), *cis,cis*-muconate lactonizing enzyme (EC 5.5.1.1; 4-carboxymethyl-4-hydroxyisocrotolactone lyase [decyclizing] [27]), and β-ketoadipate succinyl-CoA transferase (EC 2.8.3.6; 3-ketoadipate succinyl-CoA transferase [8]), except that buffer was replaced by 100 mM Tris.HCl (pH 7.5). Blanks without substrate were prepared for each extract. Protein was determined as described by Lowry et al. (22). Activities are expressed as milli-international units per milligram of protein (i.e., nanomoles per minute per milligram of protein).

***acoE* mRNA probe preparation.** Plasmid pSES38 was extracted by the rapid-extraction procedure (38). After digestion with *Sac*II, a 1,181-bp fragment from the *acoE* gene was eluted from an agarose gel with the GeneClean kit (Stratagene, La Jolla, Calif.) and used as a probe. Priming was performed by the Mega-prime random-priming method (38). DNA (20 to 50 ng) was denatured for 5 min at 100°C and incubated for 2 h with [α-^{32}P]dCTP.

Preparation of RNA. A 1.5-ml volume of culture was centrifuged for 3 min at 4°C, and the pellet was frozen in liquid nitrogen for later extraction. Extraction was performed as follows. A 1.2-ml volume of prewarmed 1.4% (wt/vol) sodium dodecyl sulfate (SDS)–4 mM EDTA was added to the frozen pellet, and the mixture was incubated at 65°C for 10 min. The tubes were then transferred into ice, and 0.6 ml of saturated NaCl was added to each tube. After 15 min, the tubes were centrifuged at 10,000 × *g* for 15 min. The aqueous phase was precipitated with 2.5 volumes of 100% ethanol, and the resulting pellet was washed twice with 70% ethanol. Treatment with RNase-free DNase I was performed for 30 min at room temperature and was followed by extraction with an equal volume of phenol at pH 5. The aqueous phase was collected, precipited, and washed with ethanol as described above. The resulting pellet was resuspended in 50 μl of water.

Northern (RNA) blot analysis. RNA was separated in formaldehyde gels and transferred to Hybond-N membrane (Amersham, Somerville, N.J.). Size was determined by migration distance as calibrated with an RNA ladder (0.24, 1.35, 2.37, 4.4, 7.46, and 9.49 kb [GIBCO/BRL]) as the standard. Prehybridization was done for 2 h at 42°C in 50% (wt/vol) formamide–5× SSPE–1× Denhardt's solution–1% (wt/vol) SDS–300 mg of tRNA per liter (1× SSPE is 0.15 M NaCl, 0.01 M NaH_2PO_4, plus 0.37% [wt/vol] EDTA [pH 7.4]; 1× Denhardt's solution is 0.2% Ficoll, 0.2% polyvinylpyrrolidone, plus 0.2% [wt/vol] bovine serum albumin). The same solution was used for hybridization with 20 to 50 ng of the *acoE* probe added. After hybridization at 42°C for 15 h, the membrane was washed twice in 2× SSPE–0.1% (wt/vol) SDS at 37°C for 20 min and then twice with 0.2× SSPE–0.1% (wt/vol) SDS at 55°C for 25 min and subjected to autoradiography (38).

Chemicals. All chemicals were analytical grade. *cis,cis*-Muconate was synthesized by the procedure described by Elvidge et al. (14), and 1,2-dihydro-1,2-dihydroxybenzoate (DHB) was prepared by using *A. eutrophus* B9 by the procedure described by Reiner (37). All other substrates, enzymes, coenzymes, and radioisotopes were obtained from Sigma Chimie, St. Quentin Fallavier, France, or C. F. Boehringer & Soehne, Mannheim, Germany.

RESULTS

Growth of *A. eutrophus* on single substrate (benzoate or acetate). In batch cultures with mineral salts medium, *A. eutrophus* 335 grew more rapidly on benzoate than on acetate. A maximum specific growth rate (μ) of 0.39 ± 0.01 h^{-1} was obtained for benzoate as compared with 0.30 ± 0.01 h^{-1} for acetate. This could be seen to be due to a more rapid specific rate of substrate consumption (q_s) in terms of carbon but also to an increased biomass yield ($Y_{X/S}$) relative to the carbon content of each substrate: q_{benzoate} = 4.1 ± 0.05 mmol/g/h (i.e., 28.7 ± 0.35 mmol of carbon per g per h) and $Y_{X/\text{benzoate}}$ = 0.54 ± 0.02 mole of carbon per mole of carbon, as opposed to q_{acetate} = 12.7 ± 0.1 mmol/g/h (i.e., 25.4 ± 0.20 mmol of carbon per g per h) and $Y_{X/\text{acetate}}$ = 0.47 ± 0.015 mole of carbon per mole of carbon.

Growth of *A. eutrophus* on benzoate-acetate mixtures. (i) Kinetic analysis. When *A. eutrophus* was grown under batch mode cultivation in media containing equimolar (20 mM) concentrations of benzoate and acetate, the behavior and substrate preference were determined by the substrate used for growth of the inoculum. Inocula pregrown on benzoate for several transfers showed diauxic growth behavior and sequential substrate consumption: benzoate followed by acetate (Fig. 2). After a brief period of accelerating growth, an exponential growth phase was observed (μ = 0.38 h^{-1}) in which only benzoate was consumed (q_{benzoate} = 4.3 ± 0.2 mmol/g/h).

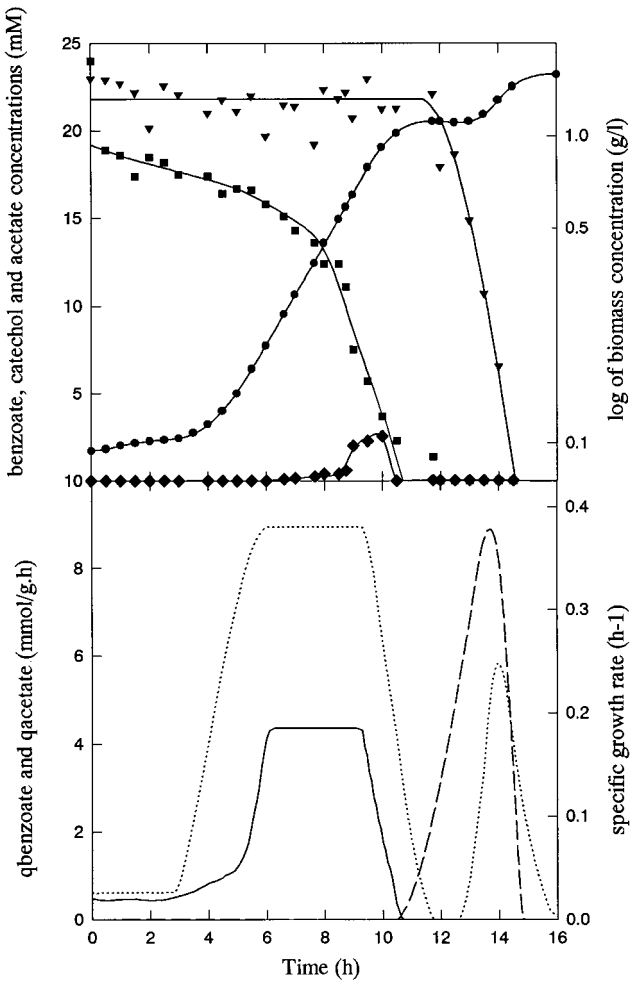

FIG. 2. Kinetics of growth and substrate consumption of *A. eutrophus* grown in batch cultures on benzoate-acetate mixture after pregrowth on benzoate. ■, benzoate concentration; ▼, acetate concentration; ◆, catechol concentration; ●, biomass; ——, q_{benzoate}; -------, q_{acetate}; · · · · ·, μ. Lines for substrates and biomass concentrations are based on interpolation (degree 4) of the raw data. Lines for μ and *q*s are directly derived from the lines for substrates and biomass.

Exponential growth was maintained until the residual benzoate concentration had fallen to approximately 5 mM, at which concentration specific rates of both growth and benzoate consumption diminished. During this exponential growth period, catechol accumulated in the medium at concentrations up to 2 mM but was later reconsumed rapidly during the period when the benzoate consumption rate began to fall. At benzoate concentrations lower than 1 mM, acetate consumption began but growth was not observed. As the acetate consumption rate increased, a second period of rapid growth was observed during which the remaining acetate was consumed. A true prolonged period of exponential growth was not seen, because of the absence of an adequate concentration of acetate (i.e., growth was still accelerating when residual substrate concentration became rate limiting), and the highest specific growth rate observed was only 0.25 h^{-1}. Specific rates of acetate consumption during this period varied in a similar manner to the growth rate and did not achieve the maximum rates observed during growth on acetate alone. This diauxic behavior was observed irrespective of the initial substrate concentrations and suggests that acetate consumption was inhibited by benzoate.

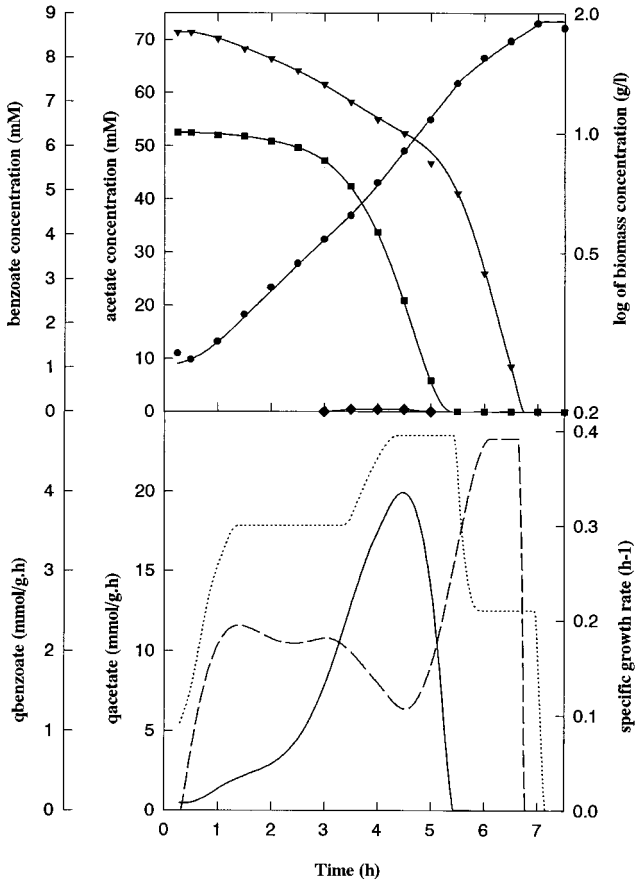

FIG. 3. Kinetics of growth and substrate consumption of *A. eutrophus* grown in batch cultures on benzoate-acetate mixture after pregrowth on acetate. ■, benzoate concentration; ▼, acetate concentration; ◆, catechol concentration; ●, biomass; ——, $q_{benzoate}$; ------, $q_{acetate}$; · · · ·, μ.

When cells pregrown on acetate were used as the inoculum (the inoculum culture was grown on 60 mM acetate without any benzoate), this diauxic growth pattern was not seen. Acetate consumption was present from the onset but diminished progressively once benzoate consumption began (results not shown). A batch culture with a modified benzoate/acetate concentration ratio (5 mM/60 mM) was used to further examine the behavior of acetate-grown cells on substrate mixtures. An initial period of exponential growth ($\mu = 0.29\ h^{-1}$) was established after inoculation, with only acetate being consumed. This first growth phase was later modified as benzoate consumption was initiated, and a second exponential growth phase ($\mu = 0.39\ h^{-1}$) was established with a specific rate of benzoate consumption of approximately 4 mmol/g/h (Fig. 3). Acetate continued to be consumed during this period of more rapid growth, but the specific consumption rate of acetate diminished progressively as an inverse function of the growth rate. Transient accumulation of trace amounts of catechol was observed during the period of rapid benzoate consumption, with rapid disappearance of the accumulated catechol once benzoate fell at a residual concentration of approximately 0.7 mM. Acetate consumption increased rapidly during this period, and a third exponential period ($\mu = 0.21\ h^{-1}$) was observed.

(ii) Enzymatic analysis. After prolonged growth (several transfers) on benzoate, no activity of either acetyl-CoA synthetase or the enzymes of the glyoxylate bypass (isocitrate lyase and malate synthase) was detected. Likewise, cells grown on acetate exhibited no activity of the enzymes of the β-ketoadipate pathway of benzoate catabolism. When benzoate-grown cells were used to inoculate the equimolar benzoate-acetate cultures described above, acetyl-CoA synthetase activity was absent during the initial phase of benzoate consumption but reached 94 mU/mg of protein in the second growth phase, in which acetate consumption occurred (the value was comparable to that assayed in cells grown on acetate alone).

In the culture containing an acetate-grown inoculum (Fig. 3), the activity of acetyl-CoA synthetase was monitored throughout the culture together with acyl-CoA synthetase activity as described by Priefert and Steinbüchel (35): similar specific activities were seen for this enzyme with either propionate or butyrate as the substrate. In addition, the activities of the three enzymes of the β-ketoadipate pathway (catechol-1,2-dioxygenase, muconate-lactonizing enzyme, and β-ketoadipate succinyl-CoA transferase) and the two enzymes of the glyoxylate bypass were measured. During the initial period of optimal acetate consumption, acetyl-CoA synthetase was present at 85 mU/mg of protein (Fig. 4) together with somewhat lower activity of the acyl-CoA synthetase (30 mU/mg of protein). During this period, the activities of the β-ketoadipate pathway enzymes were seen to increase slowly. At the beginning of the second growth phase, the enzymes specific for benzoate catabolism showed a more pronounced increase in activity which correlated with the increased rate of benzoate consumption. Acetyl-CoA synthetase, acyl-CoA synthetase, and malate synthase activities fell rapidly during this period, and the profile suggests that the synthesis of these enzymes was halted. The first-order kinetics of acetate consumption during this period was consistent with a washout effect in which the overall enzyme concentration within the culture remained constant but the specific activity diminished with cell growth. The final phase of rapid growth associated with benzoate exhaustion and metabolism of the remaining acetate saw a rapid increase in the specific activities of the enzymes necessary for acetate metabolism. The acyl-CoA synthetase reached levels significantly higher than during the initial period of acetate consumption, which may explain the particularly high rates of acetate consumption observed during this period, assuming that this enzyme can also metabolize acetate in vivo.

(iii) Northern blot analysis of *acoE* transcript. Total mRNA was isolated from samples taken throughout the culture described above (Fig. 3 and 4). On a Northern blot hybridized with an *A. eutrophus* acetyl-CoA synthetase gene *acoE*-specific probe, one RNA species of 2.4 ± 0.1 kb was detected (Fig. 5). The size of this transcript corresponds to that of the expected *acoE* gene product and is in agreement with the putative −35/−10 promoter sequence (ATGACA/CAGCAG) and the terminator proposed by Priefert and Steinbüchel (35). When *A. eutrophus* HF149 (*rpoN*) was grown on mixtures of acetate and benzoate, analogous results to those described above for strain 335 (ATCC 17697) were obtained, with acetate degradation delayed by the presence of benzoate (data not shown). Kinetic results obtained with cell samples taken from the culture described in Fig. 3 and 4 show that the transcription of *acoE* was induced during the first exponential phase of growth until t = 1.5 h. Then, the rate of transcription rapidly fell to an almost undetected level and stayed at that minimum level until the end of the second exponential phase. This halt to transcript synthesis was coincident with the appearance of β-ketoadipate pathway enzymes and a progressive increase in the specific rate of benzoate consumption. Once benzoate was totally degraded, transcription started again and high levels of mRNA were again detected. Finally, when all the acetate had been

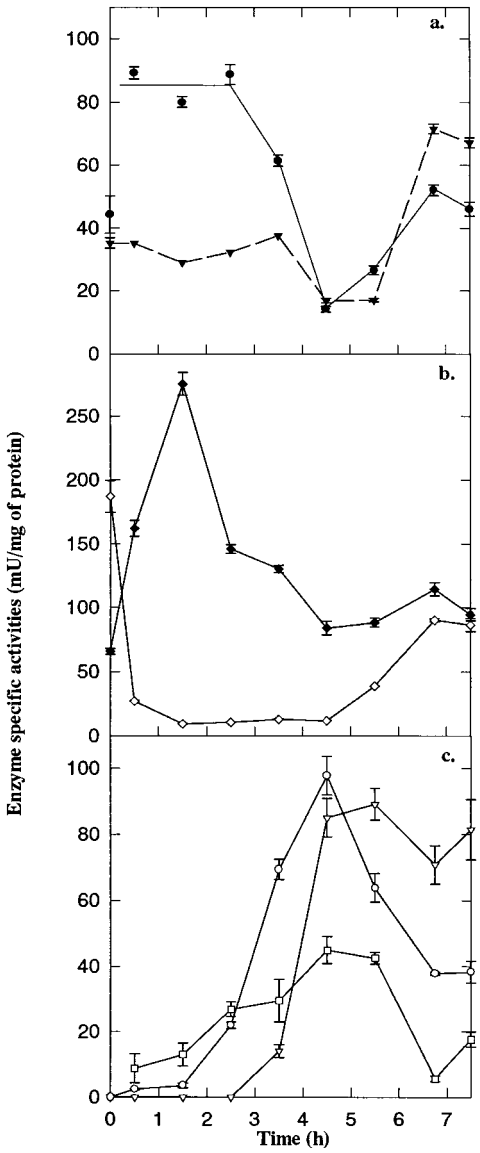

FIG. 4. Specific activity profiles of acyl-CoA synthetases (a), glyoxylic shunt enzymes (b), and some enzymes of the β-ketoadipate pathway (c) throughout the culture described for Fig. 3. (a) ●, Acetyl-CoA synthetase; ▼, acyl-CoA synthetase. (b) ◆, Malate synthase; ◇, isocitrate lyase. (c) ○, catechol-1,2-dioxygenase; ▽, muconate cycloisomerase; □, β-ketoadipate succinyl-CoA transferase. Vertical bars show the confidence intervals for the measurements, based on six repetitions.

consumed (t = 7 h), the *acoE* transcript could no longer be detected.

(iv) Chemostat study (pulse of acetate). A chemostat culture (D = 0.3 h^{-1}) of *A. eutrophus* 335 was established with benzoate as the sole carbon source and such that benzoate was the limiting substrate, i.e., residual benzoate concentration lower than the detection limit (10 μM) and considered to be zero. After the steady state was established, a pulse of acetate (60 mM) was added to the reactor to assess the possible effect of acetate on benzoate degradation. Three phases could be observed after the addition of acetate (Fig. 6). During the first phase (t = 0 to 1.6 h), the specific rate of benzoate consumption decreased slightly to 2.4 mmol/g/h and benzoate accumulated in the medium up to 2.6 mM. The specific rate of acetate consumption was constant around 13.5 mmol/g/h. During the second phase (t = 1.6 to 3 h), the specific rate of benzoate consumption rapidly increased and benzoate previously accumulated in the medium was reconsumed whereas the specific rate of acetate consumption strongly decreased to less than 5 mmol/g/h. At the same time, the biomass and specific growth rate increased to 2.3 g/liter and 0.45 h^{-1}, respectively. At t = 3.25 h, no residual substrate could be detected and steady state was slowly reestablished. At no time was catechol detected. High acyl-CoA synthetase (acetyl-, propionyl-, and butyryl-dependent) activities were measured in the chemostat before and after the pulse of acetate (Fig. 7a) with no significant change in concentration. On the other hand, activities of the enzymes of the glyoxylic shunt were very low during the benzoate-limited chemostat and increased rapidly after addition of acetate to reach 100 to 110 mU/mg before decreasing again once no residual acetate remained in the reactor (Fig. 7b). Enzymes of the β-ketoadipate pathway were low during benzoate-limited growth and were not affected directly by the addition of acetate (Fig. 7c). However, once benzoate started to accumulate in the medium, these activities increased, leading to a higher $q_{benzoate}$ and reconsumption of the accumulated substrate as shown in Fig. 6.

Identification of the key compound(s) triggering the repression. Cultures were conducted in the presence of acetate together with intermediates of the β-ketoadipate pathway (DHB, catechol, *cis*,*cis*-muconate, β-ketoadipate) or nonmetabolized analogs of the key intermediates (4-chlorobenzoate, 4-chlorocatechol, 4-methylcatechol, and *trans*,*trans*-muconate). Results show that only cultures grown in the presence of benzoate (>5 mM, in which detectable catechol accumulated), catechol, or analogs of catechol repressed the degradation of acetate (Table 1). Absence of acetate consumption during the initial growth phase was in all cases associated with a complete repression of acetyl-CoA synthetase. *cis*,*cis*-Muconate and β-ketoadipate were cometabolized with acetate, and DHB and 4-chlorobenzoate, although not supporting growth, did not repress acetate utilization.

DISCUSSION

The repression of carbon catabolism in *A. eutrophus* by the presence of a second source of carbon and energy has not been studied. Species of the closely related genera *Pseudomonas*, *P. aeruginosa* and *P. putida*, have been shown to exhibit a "catabolite repression" effect whose mechanism is not yet understood (23) but is known to be cAMP independent (32). This work

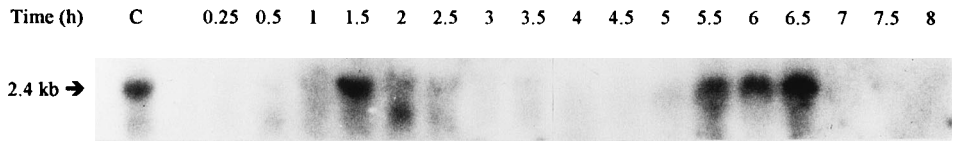

FIG. 5. Variation of the *acoE* mRNA transcript levels during a culture of *A. eutrophus* grown on a benzoate-acetate mixture (Fig. 3 and 4). Control (C) corresponds to full induction in exponential-phase acetate-grown cells. The presence of messengers was analyzed by hybridization of 10μg of total RNA with an *acoE* DNA-specific probe.

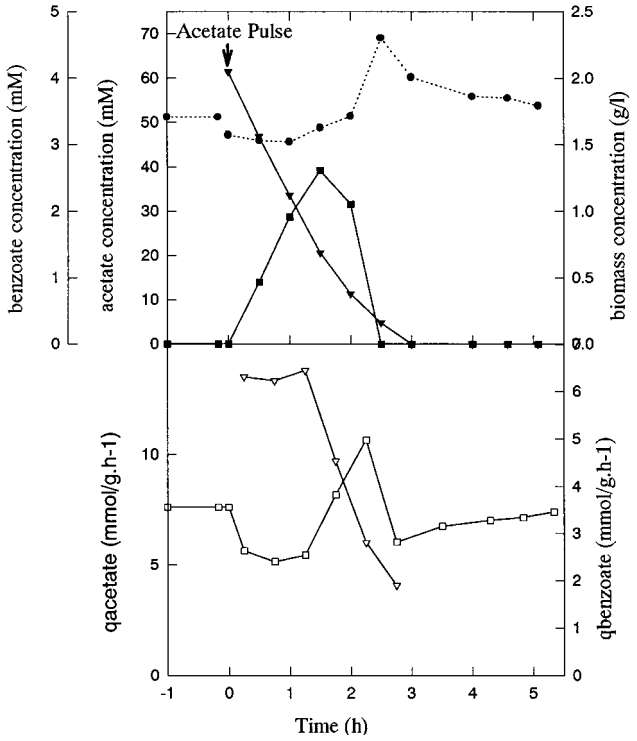

FIG. 6. Effect of a pulse of 60 mM acetate (arrow) in a benzoate-limited chemostat culture of *A. eutrophus*. ■, benzoate concentration; ▼, acetate concentration; ●, biomass; □, $q_{benzoate}$; ▽, $q_{acetate}$.

presents the first direct evidence for a catabolite repression effect in *A. eutrophus*. The synthesis of acetyl-CoA synthetase and hence the acetate metabolism were shown to be repressed during benzoate catabolism. Furthermore, mRNA analysis demonstrated that the mechanism operates at the transcriptional stage. The delay in the onset of this transcriptional control despite the presence of benzoate (Fig. 3 to 5) suggests that benzoate is not itself the repressor molecule. A close examination of the data from batch cultures indicates that the repression of acetyl-CoA synthetase occurred only after a certain level of induction of the enzymes of the β-ketoadipate pathway had occurred and benzoate consumption was under way. The study of the effect of β-ketoadipate pathway intermediates and nonmetabolized analogs revealed that catechol was the signal necessary to trigger the repression of acetyl-CoA synthetase synthesis. Catechol transiently accumulated in the medium up to 2 mM when relatively high concentrations (20 mM) of benzoate were used (Fig. 2), and traces of catechol were still found in batch cultures when lower concentrations (5 mM) of benzoate were tested (Fig. 3). In the chemostat experiment (Fig. 6 and 7), no catechol accumulated and therefore no repression of acetyl-CoA synthetase was observed. The observation that strain HF149 (*rpoN*) behaves in an identical manner confirms that *acoE* expression and regulation are sigma 54 independent and hence points toward a mechanism somewhat different from that associated with succinate (12) or glucose (18) in various pseudomonads.

While the expression of other enzymes essential for acetate metabolism (i.e., glyoxylate bypass enzymes) seems to be induced by acetate, the control of acetyl-CoA synthetase would appear to be more complex. Acetate is not the inducer, although acetyl-CoA may play this role (35). Furthermore, the distinct control of acetyl-CoA synthesis at the transcriptional level is not reinforced by a significant "catabolite inactivation" effect, and the cellular concentration of the enzyme is subject to washout due to cell proliferation. Although no transcript analysis was undertaken, the somewhat similar response of the acyl-CoA synthetase activity, shown by Priefert and Steinbüchel (35) to be a distinct enzyme from acetyl-CoA synthetase, implies that the synthesis of this enzyme may well be controlled in a similar manner during benzoate catabolism. As such, it would be interesting to compare the sequence upstream of the start codon for the structural gene encoding this enzyme with that of the *acoE* described by Priefert and Steinbüchel (35). These authors proposed a promoter region 400 bp upstream of the ribosome-binding site, confirmed in the

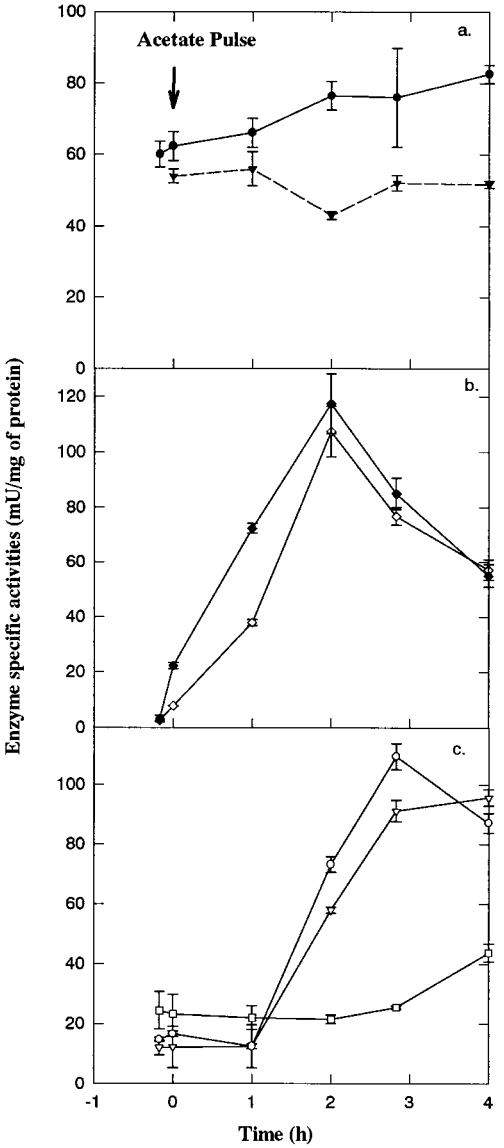

FIG. 7. Variations in enzyme specific activities for acyl-CoA synthetases (a), glyoxylic shunt enzymes (b), and enzymes of the β-ketoadipate pathway (c) during benzoate-limited chemostat culture with a pulse of acetate. The timescale corresponds to that used for Fig. 6. (a) ●, Acetyl-CoA synthetase; ▼, acyl-CoA synthetase. (b) ◆, Malate synthase; ◇, isocitrate lyase. (c) ○, Catechol-1,2-dioxygenase; ▽, muconate cycloisomerase; □, β-ketoadipate succinyl-CoA transferase. Vertical bars show the confidence intervals for the measurements, based on six repetitions.

TABLE 1. Influence of intermediates of the β-ketoadipate pathway and nonmetabolized analogs on the degradation of acetate by *A. eutrophus*[a]

Expt and compound tested (concn)	Growth pattern	Degradation of acetate	$q_{acetate}$ (mmol/g/h)	AcoA-S[b] (mU/mg of protein)
Intermediates of the β-ketoadipate pathway				
1. Benzoate (5 and 20 mM)	Diauxic	Repressed	0	0
2. DHB (2 and 5 mM)	Exponential on acetate	Not affected	12.5 ± 1.3	89.3 ± 2.0
3. Catechol (0.8 and 2 mM)	Diauxic	Repressed	0	0
4. *cis*,*cis*-Muconate (5 mM)	Cometabolism	Not affected	14.5 ± 0.9	65.7 ± 4.5
5. β-Ketoadipate (5 mM)	Cometabolism	Not affected	16 ± 1.3	78.5 ± 9.8
Nonmetabolized analogs				
6. 4-Chlorobenzoate (1 and 5 mM)	Exponential on acetate	Not affected	15 ± 1.0	88.7 ± 3.1
7. 4-Chlorocatechol (0.5 mM)	No growth	Repressed	0	ND[c]
8. 4-Methylcatechol (0.15 and 0.5 mM)	No growth	Repressed	0	ND
9. *trans*,*trans*-Muconate (0.5 and 2 mM)	Exponential on acetate	Not affected	14.7 ± 0.7	80.0 ± 1.7

[a] Results indicate the kinetic behavior for the degradation of acetate by *A. eutrophus* in the presence of both acetate and the compound tested. For experiments 1 and 3 to 5, inoculum was grown on medium containing the compound tested (respectively benzoate, catechol, *cis*,*cis*-muconate, and β-ketoadipate). For experiments 2 and 6 to 9, inoculum was grown on benzoate, as these compounds did not allow growth. The values of $q_{acetate}$ and acetyl-CoA synthetase specific activity are those obtained during the initial period of growth.

[b] AcoA-S, acetyl-CoA synthetase specific activity.

[c] ND, not determined.

present study by the size of the *acoE* transcript (2.4 ± 0.1 kb), leaving a large DNA region which may well serve as a binding region for a regulatory protein. Examination of the sequence in the proximity of the proposed promoter showed a region (AG CCATTCCCCGCGCGAGACCCGGCGCATGTGTGTG) sharing some homology with domain 1 (AGCCATACCGAT CCCGTATCGCTCGCGCTGATGGAA for *tfdR-tfdDII*) as described by Matrubutham and Harker (26) for the promoter regions of catabolic genes regulated by proteins of the LysR family in related organisms. On the other hand, no sequence homologous with the consensus sequence of catabolite gene activator protein-binding site (AA-TGAGA------TCACA-TT) (13) was seen. Although it is too early to speculate on the molecular mechanism involved, the key role played by catechol may suggest a regulatory protein whose conformation and hence binding properties are modified by catechol. Before this can be affirmed, molecular investigations of the potential binding site and other possible hairpin structures downstream of the −35/−10 region are necessary.

If mechanistic details require further study, the physiological advantages can be more clearly seen. Indeed, the growth of *A. eutrophus* on benzoate is more rapid than on acetate because of both a higher rate of carbon substrate turnover and more efficient incorporation of the substrate into the cell material. As such, the preference of this bacterium for the substrate supporting the best (i.e., most rapid) growth is coherent with the generally observed competition for substrates in which catabolite repression has been defined by Chambliss (9) as "the regulatory mechanism by which the cell coordinates metabolism of carbon and energy sources to maximise efficiency." Interestingly, one of those exceptions is that described for *P. putida*, in which benzoate has been claimed to have a repression effect on the catabolic enzymes necessary for the conversion of mandelate to benzoate, as well as the *p*-hydroxybenzoate hydroxylase (16). The authors suggested that this observation was a manner in which enzyme synthesis could be diminished, although mandelate supported more rapid growth than benzoate; however, possible competitive inhibition of transport may have been involved (31). In view of the results presented here, the influence of benzoate catabolism on the metabolism of other substrates may be more complex than was initially imagined. The transcriptional control mechanism described here appears to be specific to acetate, since other organic acids (succinate, lactate, and pyruvate) were consumed simultaneously with benzoate (1). Clearly, it would be of interest to verify whether other species able to rapidly degrade benzoate behave in a similar manner or whether the cascade regulation of the β-ketoadipate pathway of *A. eutrophus* (42) endows this organism with unique characteristics.

One of the consequences of a strict transcriptional control mechanism coupled to a relatively slow turnover of the enzyme itself is to conserve de novo protein synthesis while retaining the capacity to adapt rapidly to a fluctuating nutritional environment. Clearly, this is important in competitive environments and contributes to the metabolic versatility of this bacterium. In more general terms, the picture which is appearing regarding carbon catabolite repression mechanisms in the pseudomonads and related organisms is one of a strictly ordered hierarchy of substrate preference: organic acids repress certain enzymes of sugar degradation (44); succinate represses the degradation of certain aromatics (12); aromatic acids and acetate repress phenol degradation (19); and benzoate represses acetate catabolism (this work). Moreover, no common mechanism is implicated, and so considerable scope exists for the study of metabolic regulation of carbon substrate utilization.

ACKNOWLEDGMENTS

We thank E. Soupesne for valuable assistance in the preparation of mRNA and Northern blot analysis and M. Tisnes for his help in the synthesis of *cis*,*cis*-muconate.

We thank INRA, CNRS, and the Midi-Pyrénées regional government for financial support.

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