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Different Types of Dienelactone Hydrolase in 4-Fluorobenzoate-Utilizing Bacteria

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Of various benzoate-utilizing bacteria tested, *Alcaligenes eutrophus* 335, *A. eutrophus* H16, *A. eutrophus* JMP222, *A. eutrophus* JMP134, *Alcaligenes* strain A7, and *Pseudomonas cepacia* were able to grow with 4-fluorobenzoate as the sole source of carbon and energy. *P. cepacia* also utilizes 3-fluorobenzoate. Except for *A. eutrophus* JMP134, which is known to grow with 2,4-dichlorophenoxyacetate and 3-chlorobenzoate (R. H. Don and J. M. Pemberton, J. Bacteriol. 145:681–686, 1981), the strains were unable to grow at the expense of these compounds or 4-chlorobenzoate. Assays of cell extracts revealed that all strains express dienelactone hydrolase and maleylacetate reductase activities in addition to enzymes of the catechol branch of the 3-oxoadipate pathway when growing with 4-fluorobenzoate. Induction of dienelactone hydrolase and maleylacetate reductase apparently is not necessarily connected to synthesis of catechol 1,2-dioxygenase type II and chloromuconate cycloisomerase activities, which are indispensable for the degradation of chlorocatechols. Substrate specificities of the dienelactone hydrolases provisionally differentiate among three types of this activity. (i) Extracts of *A. eutrophus* 335, *A. eutrophus* H16, *A. eutrophus* JMP222, and *Alcaligenes* strain A7 convert *trans*-4-carboxymethylenebut-2-en-4-olide (*trans*-dienelactone) much faster than the *cis*-isomer (type I). (ii) The enzyme present in *P. cepacia* shows the opposite preference for the isomeric substrates (type II). (iii) Cell extracts of *A. eutrophus* JMP134, as well as purified dienelactone hydrolase from *Pseudomonas* strain B13 (E. Schmidt and H.-J. Knackmuss, Biochem. J. 192:339–347, 1980), hydrolyze both dienelactones at rates that are of the same order of magnitude (type III). This classification implies that *A. eutrophus* JMP134 possesses at least two different dienelactone hydrolases, one of type III encoded by the plasmid pJP4 and one of type I, which is also present in the cured strain JMP222.

Mono- and dichlorinated aromatic compounds can be degraded by bacteria in various ways, including initial conversion to chlorosubstituted catechols, followed by *ortho*-cleavage of the aromatic ring (for reviews, see references 29, 38, 40, and 41). The enzymology of this pathway has been most extensively investigated for the 3-chlorobenzoate-utilizing strain *Pseudomonas* strain B13. During growth with the chlorosubstituted compound, this strain synthesizes the following four enzymes in addition to those induced for benzoate catabolism (Fig. 1). Catechol 1,2-dioxygenase type II (EC 1.13.11.1) and muconate cycloisomerase type II (chloromuconate cycloisomerase; EC 5.5.1.7) differ from the corresponding type I enzymes in having a relaxed substrate specificity, showing high activities and affinities for chlorosubstituted substrates (10, 44). The 4-carboxymethylenebut-2-en-4-olides (dienelactones), which are generated from 2-chloro- and 3-chloro-*cis*,*cis*-muconate by cycloisomerization and simultaneous or subsequent HCl elimination, are hydrolyzed by a dienelactone hydrolase (4-carboxymethylenebut-2-en-4-olide lactonohydrolase; EC 3.1.1.45). In *Pseudomonas* strain B13 this enzyme does not show activity for 3-oxoadipate enol-lactone, the substrate for the corresponding enzyme of the ordinary 3-oxoadipate pathway (44). The fourth enzyme, maleylacetate reductase (EC 1.3.1.32), catalyzes the reduction of maleylacetate to 3-oxoadipate, which is the first common intermediate of the ordinary and the modified *ortho*-cleavage pathways (38).

The catabolism of 4-fluorobenzoate (4FB) by *Pseudomonas* strain B13 was proposed by Schreiber et al. (46) to proceed via 4-fluorocatechol and 3-fluoro-*cis*,*cis*-muconate to a supposedly unstable fluoromuconolactone, which by spontaneous HF elimination should yield the *cis*-dienelactone (Fig. 1). This intermediate could be further converted into 3-oxoadipate by subsequent action of dienelactone hydrolase and maleylacetate reductase. Schreiber et al. (46) did not find catechol 1,2-dioxygenase type II or chloromuconate cycloisomerase activities in 4FB-grown cells. Therefore, for the degradation of 4FB, *Pseudomonas* strain B13 seemed to need only dienelactone hydrolase and maleylacetate reductase in addition to the enzymes for benzoate catabolism.

If these observations also apply to other bacteria, the growth of benzoate-utilizing strains with 4FB should indicate the presence of dienelactone hydrolase and maleylacetate reductase independent of the ability to induce catechol 1,2-dioxygenase type II and chloromuconate cycloisomerase. Since the occurrence of enzymes of the modified *ortho*-pathway independently of each other could be of interest for the evolution of this degradative route, we checked several benzoate-utilizing strains for their ability to grow with 4FB and assayed some of the enzyme activities induced.

(The results presented here have been reported previously in a preliminary form [M. Schlömann, E. Schmidt, and H.-J. Knackmuss, Syst. Appl. Microbiol. 5:259, 1984; M. Schlö-

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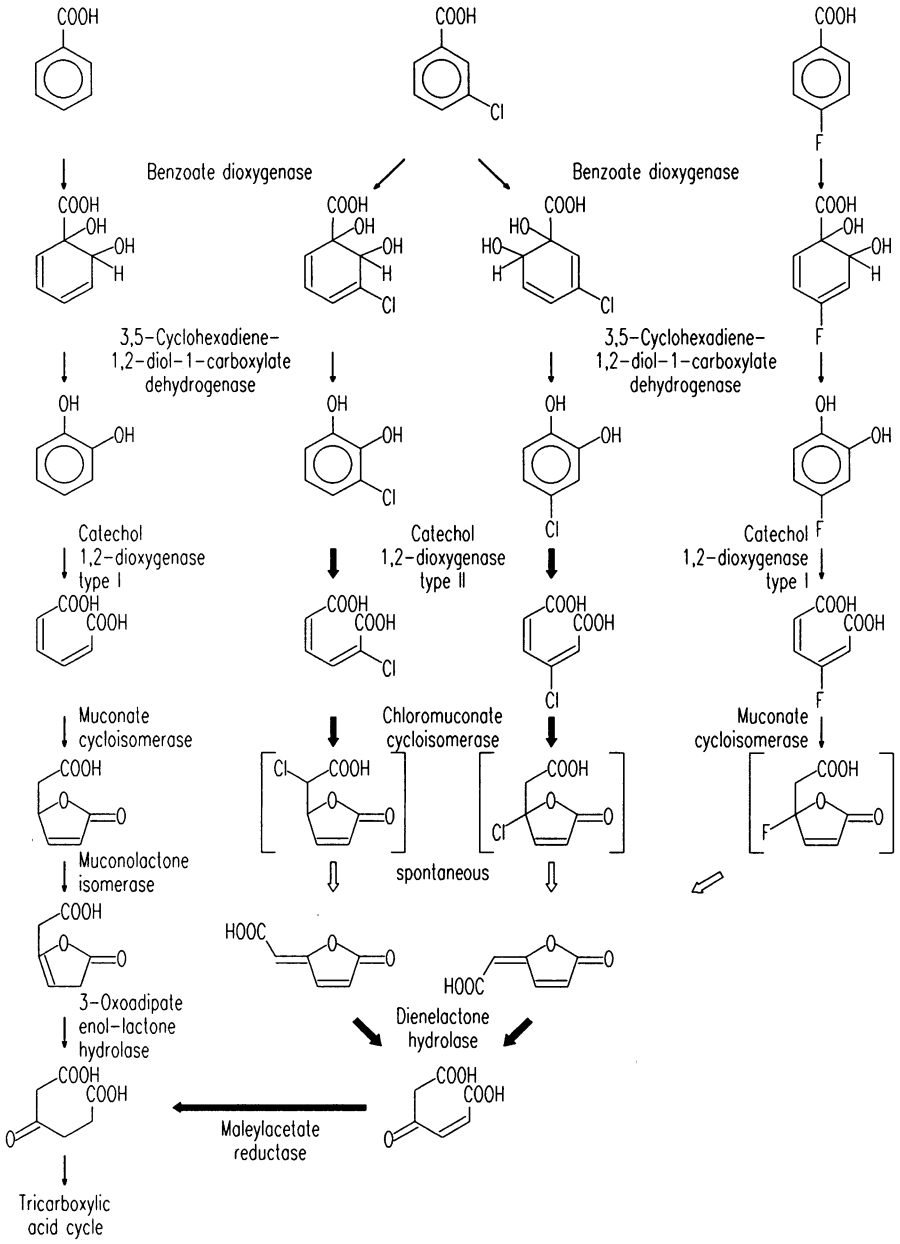

FIG. 1. Pathways for the degradation of benzoate, 3-chlorobenzoate, and 4FB in *Pseudomonas* strain B13 as described by Schreiber et al. (46), Schmidt and Knackmuss (44), and Reineke (38). Symbols: →, enzymes for benzoate degradation via the 3-oxoadipate pathway; ➡, enzymes induced additionally during growth with 3-chlorobenzoate; ⇨, spontaneous reactions. The 4FB pathway shown here does not take into account the results in the accompanying paper (42).

mann, K.-L. Ngai, H.-J. Knackmuss, and L. N. Ornston, Fed. Proc. **44**:471, 1985].)

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this investigation are listed in Table 1. They were grown in the minimal medium described by Dorn et al. (8), the buffer concentration being increased twofold (20). The media were supplemented with the indicated carbon source, usually at 2 or 5 mM. Media were solidified, when needed, by addition of 1.4% agar. All cultures were grown aerobically at 30°C, liquid cultures in baffled Erlenmeyer flasks on rotary shakers at 100 rpm. Stock cultures were maintained on agar plates containing benzoate, except for *Alcaligenes eutrophus* JMP134, which was subcultured with 2,4-dichlorophenoxyacetate.

Investigation of carbon source utilization. Growth on solid media was evaluated after the strains had been incubated for up to 4 weeks with the carbon source at an initial concentration of 2 mM. Doubling times for growth in liquid culture were derived from growth curves obtained by turbidity measurements in a Klett-Summerson photoelectric colorimeter.

Preparation of cell extracts. Cells were harvested by centrifugation during late exponential growth and suspended in 50 mM Tris hydrochloride buffer (pH 7) containing 4 mM

TABLE 1. Strains utilizing 4FB as the sole source of carbon and energy

Organism[a]	Reference
Utilization of 4FB[b]:	
A. eutrophus 335 (ATCC 17697, DSM 531)	5, 24
A. eutrophus H16 (ATCC 17699, DSM 428)	5
A. eutrophus JMP134	35
A. eutrophus JMP222	35
Alcaligenes strain A7	47
P. cepacia (ATCC 17759, DSM 50181)	51
No utilization of 4FB:	
Acinetobacter calcoaceticus (ATCC 11171, DSM 590)	1
A. faecalis (ATCC 15554, DSM 30033)	28
A. paradoxus (ATCC 17713, DSM 66)	5
Arthrobacter strain 25/1 (DSM 20389)	11
Azotobacter chroococcum (DSM 377)	37
P. acidovorans (ATCC 17406, DSM 50053)	51
P. aureofaciens (ATCC 17415, DSM 50139)	51
P. chlororaphis (ATCC 9446, DSM 50083)	51
P. fluorescens (ATCC 17581, DSM 50124)	51
P. putida A3.12 (ATCC 12633, DSM 50202)	51
P. testosteroni (ATCC 17407, DSM 50242)	51

[a] All strains for which a DSM number is given were obtained from Deutsche Sammlung von Mikroorganismen, Gesellschaft für Biotechnologische Forschung, Braunschweig-Stöckheim, Federal Republic of Germany.

[b] Growth was evaluated after incubation on agar with 2 mM 4FB for up to 4 weeks. Growth on benzoate agar served as positive control, and growth on mineral agar without a carbon source served as a negative control.

$MnSO_4$. After addition of a trace of DNase I, the cells were disrupted with an Aminco French press. Whole cells and cell debris were removed by centrifugation at 100,000 × *g* for 1 h at 5°C.

Enzyme assays. All enzyme assays were performed by spectrophotometric methods; 1-ml quartz cuvettes at 25°C were used. An activity of 1 enzyme unit (1 U) catalyzes the conversion of 1 μmol of substrate per min. Catechol 1,2-dioxygenase was assayed by a modification of the procedure of Hegeman (21). The cuvettes initially contained 30 mM Tris hydrochloride buffer (pH 8), 1.2 mM EDTA, and the extract to be tested. The reaction was started after 4 min by the addition of 0.3 mM catechol or 3-chlorocatechol. The activity with the latter substrate served to distinguish between catechol 1,2-dioxygenase types I and II (9). Extinction coefficients were as reported earlier (10). The activity of protocatechuate 3,4-dioxygenase was measured as described by Stanier and Ingraham (50). Activities of muconate cycloisomerase, chloromuconate cycloisomerase (muconate cycloisomerase type II), and dichloromuconate cycloisomerase were determined by modifications of published procedures (27, 33, 44). The assays were performed by using 30 mM Tris hydrochloride (pH 8) containing 1 mM $MnCl_2$ and 1.0 mM *cis,cis*-muconate, 2-chloro-*cis,cis*-muconate, or 2,4-dichloro-*cis,cis*-muconate. Since the assays were done with crude cell extracts, dienelactone hydrolase was not added as an auxiliary enzyme. 3-Oxoadipate enol-lactone hydrolase was assayed by the method of Ornston (32) in the presence of 30 mM Tris hydrochloride (pH 8), 0.67 mM (+)-muconolactone, and nonlimiting amounts of muconolactone isomerase from *Pseudomonas putida* PRS2000. For the determination of dienelactone hydrolase activity, the method of Schmidt and Knackmuss (44) was modified so that the assay mixture contained 10 mM histidine hydrochloride (pH 6.5) and 0.1 mM *cis*- or *trans*-4-carboxymethylenebut-2-en-4-olide (dienelactone). The extinction coefficients used were 15,625 M^{-1} cm^{-1} for conversion of the *trans* isomer (44) and 17,000 M^{-1} cm^{-1} for the *cis* isomer, the extinction of the product maleylacetate at 280 nm being neglected. A modification of the procedure of Gaal and Neujahr (16) was used to measure maleylacetate reductase activity. The extract was added to cuvettes containing 0.2 mM NADH in 50 mM Tris hydrochloride (pH 7.5). After nonspecific NADH oxidation at 340 nm had been recorded for 2 min, maleylacetate was added to a final concentration of 0.1 mM and recording of NADH oxidation was continued. The activity of maleylacetate reductase was calculated from the difference of the two oxidation rates. In some cases activity with NADPH as cosubstrate was also assayed. The protein concentration in cell extracts was estimated as described by Bradford (2), with the modification that less dye was used to prepare the reagent (80 mg of Coomassie brilliant blue G250; E Merck AG, Darmstadt, Federal Republic of Germany). Bovine serum albumin served as the standard.

Chemicals. 3-Chlorocatechol (46), *cis,cis*-muconate (45), 2-chloro-*cis,cis*-muconate (45), and the isomeric dienelactones *cis*- and *trans*-4-carboxymethylenebut-2-en-4-olide (39) were available from previous syntheses. 2,4-Dichloro-*cis,cis*-muconate was prepared as described elsewhere (27). Maleylacetate was prepared on the day of its use from *trans*-4-carboxymethylenebut-2-en-4-olide either by alkaline hydrolysis (14) or by use of partially purified dienelactone hydrolase (44). (+)-Muconolactone and purified muconolactone isomerase from *P. putida* PRS2000 were kindly supplied by K.-L. Ngai and L. N. Ornston.

RESULTS

Utilization of 4FB as a growth substrate. Several benzoate-assimilating strains, representing predominantly *Alcaligenes* and *Pseudomonas* species, were arbitrarily chosen and tested for growth with 4FB on solid media (Table 1). Of those investigated, six strains formed colonies in the presence of 2 mM 4FB as the sole source of carbon and energy. During subcultivation of these organisms in 4FB-containing liquid media, a more or less pronounced adaptation to the new growth substrate 4FB was observed. Cultures of *A. eutrophus* 335 and *A. eutrophus* H16, which had not been exposed to 4FB before, showed doubling times of about 14 h with 2 or 5 mM 4FB, whereas after 15 generations doubling times between 4 and 6 h were obtained with 5 mM 4FB. Higher substrate concentrations (10 or 15 mM) resulted in significantly slower growth. The doubling time of *Alcaligenes* strain A7 decreased within six generations from 25 h (2 mM 4FB) to 15 h (5 mM 4FB). *Pseudomonas cepacia* (initial doubling time, 26 to 28 h with 2 mM 4FB), after adaptation for 10 generations, grew with a doubling time of 8 h (5 mM 4FB). Growth of unadapted cultures of *A. eutrophus* JMP134(pJP4) was significantly inhibited by high concentrations of 4FB (at 2 mM the doubling time was 7 h; at 5 mM it was ≥30h). After subcultivation for 16 generations this strain showed a doubling time of about 4 h at a substrate concentration of 5 mM. This adaptation appears to be correlated with the presence of pJP4, since doubling times of the cured *A. eutrophus* JMP222 (30 to 40 h at 2 mM 4FB) did not decrease during prolonged subcultivation on 4FB.

Utilization of other carbon sources. Strains that grew with 4FB (Table 1) were also tested for their ability to grow with 3-fluorobenzoate, 3-chlorobenzoate, 4-chlorobenzoate, 2,4-dichlorophenoxyacetate, and resorcinol. On plates with 3-fluorobenzoate only *P. cepacia* developed colonies. With the exception of *A. eutrophus* JMP134, which is known to

TABLE 2. Enzyme activities in cell extracts of *A. eutrophus* 335 after growth with different substrates

Enzyme and substrate (cosubstrate)	Sp act (U/mg of protein) after growth with:			
	4FB	Benzoate	4-Hydroxybenzoate	Succinate
Catechol 1,2-dioxygenase				
Catechol	1.3	0.50	0.017	<0.002
3-Chlorocatechol	<0.002	<0.002	<0.002	ND[a]
Muconate cycloisomerase				
cis,cis-Muconate	2.4	1.7	<0.002	<0.002
2-Chloro-*cis,cis*-muconate	<0.01	ND	ND	ND
3-Oxoadipate enol-lactone hydrolase	4.1	3.1	2.3	<0.2
Dienelactone hydrolase				
trans-Dienelactone	4.6	0.025	0.021	0.031
cis-Dienelactone	0.01	<0.002	<0.002	<0.002
Maleylacetate reductase				
Maleylacetate (NADH)	0.44	0.043	0.036	0.015
Maleylacetate (NADPH)	0.38	0.013	0.025	ND
Protocatechuate-3,4-dioxygenase	<0.02	<0.02	0.72	ND

[a] ND, Not determined.

grow with 3-chlorobenzoate and 2,4-dichlorophenoxyacetate (6), the strains did not utilize the chlorosubstituted substrates. None of the strains grew with resorcinol.

Enzyme activities induced during growth with 4FB. To investigate the induction of enzymes possibly involved in 4FB degradation, we prepared cell extracts from the 4FB-utilizing strains after growing them on various substrates. For some strains subcultivation with 4FB increased the level of uninduced enzyme activities (see below). Therefore, if not otherwise stated, preexposure to this substrate was allowed only for cultures that were used to determine enzyme levels during growth with 4FB.

In 4FB-grown cells of *A. eutrophus* 335, enzymes for benzoate degradation via the 3-oxoadipate pathway, as well as dienelactone hydrolase and maleylacetate reductase, were clearly induced (Table 2). The rate of hydrolysis of the *trans*-dienelactone (*trans*-4-carboxymethylenebut-2-en-4-olide) was much higher than that of the *cis*-isomer. Activities for the 1,2-dioxygenation of 3-chlorocatechol or for the cycloisomerization of 2-chloro-*cis,cis*-muconate could not be detected. Protocatechuate 3,4-dioxygenase was induced during the growth of *A. eutrophus* 335 with 4-hydroxybenzoate, but not with 4FB. Similar activity levels to those in *A. eutrophus* 335 were also found in *A. eutrophus* H16 (data not shown). After prolonged subcultivation with 4FB (15 generations), both strains exhibited considerable dienelactone hydrolase activities in cell extracts from benzoate-grown cells (0.6 U/mg of protein in *A. eutrophus* 335; 1.2 U/mg of protein in *A. eutrophus* H16).

Like *A. eutrophus* 335, *Alcaligenes* strain A7 induced catechol 1,2-dioxygenase, muconate cycloisomerase, and 3-oxoadipate enol-lactone hydrolase during growth with 4FB (Table 3). Activities of dienelactone hydrolase and maleylacetate reductase in all three extracts were very low but clearly detectable. The *cis*-dienelactone was not hydrolyzed at a significant rate.

In 4FB-grown cells of *P. cepacia* a *cis*-dienelactone-hydrolyzing activity and a maleylacetate reductase were induced in addition to enzymes for benzoate catabolism via the 3-oxoadipate pathway (Table 4). However, there was no

TABLE 3. Enzyme activities in cell extracts of *Alcaligenes* strain A7 after growth with different substrates

Enzyme and substrate	Sp act (U/mg of protein) after growth with:		
	4FB	Benzoate	Succinate
Catechol 1,2-dioxygenase	0.52	0.81	<0.001
Muconate cycloisomerase	1.9	0.78	<0.01
3-Oxoadipate enol-lactone hydrolase	0.51	0.54	0.04
Dienelactone hydrolase			
trans-Dienelactone	0.055	0.010	0.047
cis-Dienelactone	<0.001	<0.001	ND[a]
Maleylacetate reductase	0.045	0.017	0.008

[a] ND, Not determined.

substantial activity for the hydrolysis of the *trans*-dienelactone. As in *A. eutrophus* 335, protocatechuate 3,4-dioxygenase was detected in 4-hydroxybenzoate-grown, but not in 4FB-grown, cells.

Cultures of *A. eutrophus* JMP134 that were not fully adapted to 4FB showed no induction of catechol 1,2-dioxygenase type II or dichloromuconate cycloisomerase when grown with 4FB (Table 5). The small amount of 3-chlorocatechol-dioxygenating activity can be attributed to the catechol 1,2-dioxygenase type I (36). In these cells dienelactone hydrolase was present, but at lower levels than in 2,4-dichlorophenoxyacetate-grown cells. However, after growth with this substrate, cultures adapted to 4FB exhibited activities for the 1,2-dioxygenation of 3-chlorocatechol as high as 0.66 U/mg of protein (1.9 U/mg with catechol) and activities for the hydrolysis of the *trans*-dienelactone of up to 5.4 U/mg. Extracts from 2,4-dichlorophenoxyacetate-grown cells, as well as from 4FB-grown cells, hydrolyzed the *cis*-dienelactone somewhat more slowly than the *trans*-isomer, both rates still being of the same order of magnitude.

A. eutrophus JMP222, a cured derivative of JMP134 (35), also did not induce any catechol 1,2-dioxygenase type II or dichloromuconate cycloisomerase during growth with 4FB, whereas dienelactone hydrolase and maleylacetate reductase were present (Table 6). Interestingly, the *trans*-dienelactone was hydrolyzed about 36-fold faster than the *cis*-isomer, this ratio being significantly higher than that in JMP134.

DISCUSSION

The utilization of 4FB as the sole source of carbon and energy has been described earlier for several bacterial gen-

TABLE 4. Enzyme activities in cell extracts of *P. cepacia* after growth with different substrates

Enzyme and substrate	Sp act (U/mg of protein) after growth with:			
	4FB	Benzoate	4-Hydroxybenzoate	Succinate
Catechol 1,2-dioxygenase	1.1	0.43	0.006	0.005
Muconate cycloisomerase	0.71	0.68	<0.002	<0.002
3-Oxoadipate enol-lactone hydrolase	0.29	1.9	3.8	0.10
Dienelactone hydrolase				
trans-Dienelactone	0.002	<0.001	<0.001	<0.001
cis-Dienelactone	0.065	<0.001	<0.001	<0.001
Maleylacetate reductase	0.11	0.019	0.004	<0.002
Protocatechuate 3,4-dioxygenase	<0.01	<0.01	0.55	ND[a]

[a] ND, Not determined.

TABLE 5. Enzyme activities in cell extracts of *A. eutrophus* JMP134 after growth with different substrates

Enzyme and substrate (cosubstrate)	Sp act (U/mg of protein) after growth with:			
	2,4-Dichlorophenoxyacetate	4FB[a]	Benzoate	Succinate
Catechol 1,2-dioxygenase				
Catechol	0.74	3.8	0.46	<0.01
3-Chlorocatechol	0.34	0.06	0.03	<0.01
Muconate cycloisomerase				
cis,cis-Muconate	0.04	4.6	1.5	<0.01
2,4-Dichloro-*cis,cis*-muconate	0.72	<0.01	<0.01	<0.01
3-Oxoadipate enol-lactone hydrolase	0.25	0.42	1.8	0.01
Dienelactone hydrolase				
trans-Dienelactone	2.0	0.53	0.18	0.11
cis-Dienelactone	1.4	0.29	0.12	0.06
Maleylacetate reductase				
Maleylacetate (NADH)	0.21	0.14	0.04	0.03
Maleylacetate (NADPH)	0.07	0.07	0.05	0.05

[a] The crude extract was prepared from cells which, after subcultivation with 2,4-dichlorophenoxyacetate, had been grown with 4FB for seven generations.

era including *Pseudomonas* (19, 31, 46, 52), *Paracoccus* (26), *Acinetobacter* (53), *Corynebacterium* (22, 23), *Alcaligenes* (31), and *Aureobacterium* (31). During the present investigation, which focused on bacteria that were known to degrade aromatic compounds, several *A. eutrophus* strains, *Alcaligenes* strain A7, and *P. cepacia* were found to be able to grow with 4FB. The last-mentioned bacterium also utilizes 3-fluorobenzoate, thus having a capability which until now has been described only for *Paracoccus denitrificans* (26), two *Alcaligenes* strains (31), and a 3-fluorobenzoate-enriched bacterial strain, FLB300 (12).

4FB generally seems to be degraded by bacteria via 1-carboxy-1,2-dihydroxy-4-fluoro-3,5-cyclohexadiene, 4-fluorocatechol, and 3-fluoro-*cis,cis*-muconate (Fig. 1). This route has been established with 4FB-cooxidizing strains as well as with 4FB-utilizing strains (3, 4, 19, 23, 31, 46, 49). In contrast, the 4FB degradation via 4-hydroxybenzoate and protocatechuate, which was recently observed in *Aureobacterium* strain RHO25 (31), presumably is an exception. The induction of catechol 1,2-dioxygenase and muconate cycloisomerase by all 4FB-utilizing strains investigated in this study suggests that they use the catechol branch of the 3-oxoadipate pathway for the degradation of 4FB. Moreover, *A. eutrophus* 335 and *P. cepacia*, the strains for which the 4-hydroxybenzoate degradation had been shown previously to involve *ortho*-cleavage of protocatechuate (25, 34), did not show any protocatechuate 3,4-dioxygenase activity when grown with 4FB. Therefore, for these bacteria a 4FB catabolism via 4-hydroxybenzoate and protocatechuate can clearly be excluded.

TABLE 6. Enzyme activities in cell extracts of *A. eutrophus* JMP222 after growth with different substrates

Enzyme and substrate (cosubstrate)	Sp act (U/mg of protein) after growth with:		
	4FB	Benzoate	Succinate
Catechol 1,2-dioxygenase			
Catechol	1.3	0.62	0.03
3-Chlorocatechol	<0.002	<0.002	<0.002
Muconate cycloisomerase			
cis,cis-Muconate	2.3	1.0	0.03
2-Chloro-*cis,cis*-muconate	0.03	<0.01	ND[a]
2,4-Dichloro-*cis,cis*-muconate	<0.01	<0.01	<0.01
3-Oxoadipate enol-lactone hydrolase	1.1	1.1	<0.2
Dienelactone hydrolase			
trans-Dienelactone	0.18[b]	0.012	0.011
cis-Dienelactone	0.005[b]	<0.001	<0.001
Maleylacetate reductase			
Maleylacetate (NADH)	0.16	0.035	0.014
Maleylacetate (NADPH)	0.14	0.029	0.017

[a] ND, Not determined.

[b] After growth of the cells with 4FB some extracts showed an activity with *trans*-dienelactone of only 0.05 or even 0.01 U/mg. The rate with *cis*-dienelactone in these extracts was at the detection limit.

3-Fluoro-*cis,cis*-muconate was reported by Schreiber et al. (46) to be further converted to maleylacetate via *cis*-dienelactone, supposedly the product of a nonenzymatic elimination of HF from 4-fluoromuconolactone. Initially assuming this pathway to be correct, we investigated whether dienelactone hydrolase and maleylacetate reductase activities were induced in strains growing with 4FB. Indeed, apart from low activities in *Alcaligenes* strain A7, all 4FB-utilizing strains examined here induced considerable levels of dienelactone hydrolase and maleylacetate reductase activities during growth with 4FB, whereas much lower levels were expressed in benzoate-grown and succinate-grown cells. This induction pattern was clearly different from that of the 3-oxoadipate enol-lactone hydrolases, proving that the respective dienelactone hydrolase activities do not result (or result only to a very small extent) from low substrate specificities of 3-oxoadipate enol-lactone hydrolases. In addition, the induction was in accordance with the 4FB-degradative pathway of Schreiber et al. (46), seemingly implying a role for dienelactone hydrolases and maleylacetate reductase in 4FB catabolism.

Although this conclusion probably is correct for the reductase, it need not be true for the dienelactone hydrolases. In fact, the accompanying paper (42) clearly states that 4-fluoromuconolactone, and not the *cis*-dienelactone, is the major lactonic intermediate in this catabolic route. In addition, that paper presents preliminary evidence that 4-fluoromuconolactone might be converted by 3-oxoadipate enol-lactone hydrolases. Consequently, the role of dienelactone hydrolases in 4FB degradation might be restricted to an auxiliary function in 4-fluoromuconolactone hydrolysis or to conversion of a defluorinated byproduct, or they might even be induced without having any function in this pathway. Which of these or other possibilities applies can be decided only after chromatographic separation of the respective hydrolases and kinetic analysis of their ability to convert 4-fluoromuconolactone. Preliminary results of experiments addressing these questions show significant differences among the strains under investigation.

Dienelactone hydrolase activities have previously been reported in connection with the degradation of chloroaromatic compounds (13, 14, 17, 36, 44, 48). However, except for *A. eutrophus* JMP134, which is known to utilize 2,4-dichlorophenoxyacetate and 3-chlorobenzoate (6), characteristically none of the 4FB-degrading strains investigated in this study was able to grow with 3-chlorobenzoate, 4-chlorobenzoate, or 2,4-dichlorophenoxyacetate. Correspondingly, neither *A. eutrophus* 335 nor *A. eutrophus* JMP222 induced any chlorocatechol-dioxygenating or chloromuconate-cycloisomerizing activity during growth with 4FB.

TABLE 7. Substrate specificities of different dienelactone hydrolases

Strain	Sp act (U/mg of protein) with[a]:		Sp act ratio (*trans*/*cis*)	Type of dienelactone hydrolase
	trans-Dienelactone	*cis*-Dienelactone		
A. eutrophus 335	4.6	0.01	460	I
A. eutrophus H16	2.0	0.006	330	I
Alcaligenes strain A7	0.055	<0.001	>55	I
A. eutrophus JMP222	0.18	0.005	36	I
P. cepacia	0.002	0.065	0.03	II
A. eutrophus JMP134	0.53	0.29	1.8	III
Pseudomonas strain B13	ND[b]	ND	4.0[c]	III

[a] The specific activities in 4FB-grown cells were taken from Tables 2 to 6, and those for *A. eutrophus* H16, were determined under the same conditions.
[b] ND, Not determined.
[c] Value obtained with partially purified dienelactone hydrolase (44) at a substrate concentration of 0.1 mM.

Therefore it can be concluded that some bacteria harbor dienelactone hydrolase and maleylacetate reductase activities, while lacking the other two enzymes necessary for complete chlorocatechol degradation. The relevance of this finding for the evolution of a chlorocatechol-oxidizing pathway still has to be evaluated.

Enzyme assays with cell extracts showed that dienelactone hydrolases with significantly different substrate specificities are expressed by 4FB-utilizing bacteria. The data summarized in Table 7 provisionally differentiate among three types of this enzyme activity. Extracts from *A. eutrophus* 335, *A. eutrophus* H16, *A. eutrophus* JMP222, and *Alcaligenes* strain A7 hydrolyzed the *trans*-dienelactone considerably faster than the *cis*-dienelactone (dienelactone hydrolase type I). An opposite preference for the two dienelactone isomers was observed in *P. cepacia* (dienelactone hydrolase type II). Extracts from *A. eutrophus* JMP134 hydrolyzed *cis*- and *trans*-dienelactone with rates of the same order of magnitude. The enzyme responsible for this substrate specificity thus resembles the dienelactone hydrolase (type III), which was isolated from *Pseudomonas* strain B13 (30, 44). At substrate saturation the B13 enzyme hydrolyzes both dienelactones at about the same rate (44), whereas at 0.1 mM, as used in the enzyme assays, it hydrolyzes the *trans*-dienelactone four times faster than the *cis*-dienelactone, because of a more favorable K_m value for the former.

The objection could be raised against this differentiation that an apparent type III dienelactone hydrolase activity is simulated by a mixture of type I and type II enzymes in the same extract. This argument can be easily invalidated for the hydrolase from *Pseudomonas* strain B13, because this enzyme was shown to hydrolyze both dienelactones, even after it had been purified to homogeneity (30). Further investigation proved that this is also true for the pJP4-encoded dienelactone hydrolase from *A. eutrophus* JMP134 (data not shown). In addition to the above objection, it might be asked whether the ratios of hydrolysis rates given in Table 7 for the dienelactone hydrolases of *Pseudomonas* strain B13 (ratio of 4) and *A. eutrophus* JMP222 (ratio of 36) are sufficiently different to justify an assignment to separate types of the enzyme. Clearly, the classification demands more detailed kinetic analyses and physical evidence. Results from purification and characterization of several dienelactone hydrolases, which are to be published separately (for preliminary reports: see reference 43 and M. Schlömann, K.-L. Ngai, H.-J. Knackmuss, and L. N. Ornston, Fed. Proc. **44**:471, 1985), indeed show that the classification made here only on the basis of substrate specificity is valid and useful for the further investigation of these enzymes.

The apparent presence of different types of dienelactone hydrolase in *A. eutrophus* JMP134 and in its cured derivative *A. eutrophus* JMP222 implies that JMP134 possesses (at least) two dienelactone hydrolases, one of type III encoded by the plasmid pJP4 and another of type I encoded by the chromosome or by a megaplasmid (15). The type III enzyme has been observed previously (36), and its gene has already been localized on pJP4 (7, 18). In contrast, the existence of the type I dienelactone hydrolase in JMP134 has not been reported until now. The occurrence of these two enzymes in addition to probably two 3-oxoadipate enol-lactone hydrolases makes JMP134 a very promising object for evolutionary studies.

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