Regulation of catabolic pathways of phenoxyacetic acids and phenols in Alcaligenes eutrophus JMP 134

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Abstract. *Alicaligenes eutrophus* JMP 134 is able to grow on 2,4-dichloro-, 4-chloro-2-methyl- and 2-methylphenoxy acetic acid. The unsubstituted phenoxyacetic acid, however, is no growth substrate due to very poor induction of the 2,4-D monooxygenase. Spontaneous mutants of *Alcaligenes eutrophus* JMP 134 capable of growth with phenoxyacetic acid were selected on agar plates. One of these mutants, designated *Alcaligenes eutrophus* JMP 134-1, shows constitutive production of six enzymes of the 2,4-D pathway, which were known to be localized in at least three different transcriptional units. A common regulatory gene is postulated to be mutated. 2,4-Dichloro-, 4-chloro-2-methyl- and 2-methylphenoxyacetic acid were the inducers of the enzymes of the "chloroaromatic pathway" in *Alcaligenes eutrophus* JMP 134. Phenol and 2-methylphenol, metabolites of the degradation of phenoxyacetic acid and 2-methylphenoxyacetic acid, were shown to be inducers of the meta-cleavage pathway, whereas 2,4-dichlorophenol and 4-chloro-2-methylphenol were not. Thus efficient regulation prevents chloroaromatics from being misrouted into the unproductive meta-cleavage pathway. Because 2,4-dichloro- and 4-chloro-2-methylphenol did not show any induction potential, they were growth substrates only for the mutant strain JMP 134-1.

Key words: Phenoxyacetic acids — Phenols — *Alcaligenes eutrophus* JMP 134 — Ortho-cleavage pathways — Meta-cleavage pathway — Regulation

Genes coding for the catabolism of 2,4-dichlorophenoxy acetic acid (2,4-D) by *Alcaligenes eutrophus* JMP 134 have been located on the plasmid pJP4 (Don et al. 1985; Streber et al. 1987). Studies on the substrate specific of enzymes for the catabolism of 2,4-D, 4-chloro-2-methylphenoxyacetic acid (MCPA) and 2-methylphenoxyacetic acid (2MPA) indicated that isoenzymes function for certain catabolic steps (Pieper et al. 1988). 2,4-D and MCPA seems to be metabolized by common enzymes of the ortho-cleavage pathway specialized for the turnover of chlorinated compounds. In contrast 2MPA, after conversion to the corresponding catechol, is channeled into the ortho- as well as into an also existing meta-cleavage pathway (Pieper et al. 1988). Obviously an effective regulation prevents the induction of meta-cleavage during growth on chlorosubstituted phenoxyacetic acids. Whereas various research groups investigated the regulation of the 3-oxoadipate pathway in different bacterial species (Ornston 1966; Canovas and Stanier 1967; Kemp and Hegeman 1968; Rann and Cain 1969; Johnson and Stanier 1971) and of the meta-cleavage pathway (Feist and Hegeman 1969; Sala-Trepat et al. 1972; Murray and Williams 1974; Bayly et al. 1977; Hughes and Bayly 1983), there is only scare information concerning the regulation of chloroaromatic pathways (Don et al. 1985; Ghosal et al. 1985; Streber et al. 1987; Frantz and Chakrabarty 1987).

It has been reported (Pieper et al. 1988) that out of eleven variously substituted phenoxyacetic acids only 2,4-D, MCPA and 2MPA served as growth substrates for *Alcaligenes eutrophus* JMP 134. Although unsubstituted phenoxyacetic acid (PA) was not a growth substrate, derivative strains capable of growth on this compound occurred with high frequency. This extension of substrate range was shown to be due to a mutation on the plasmid pJP4 (Pemberton et al. 1979). To clarify the nature of this transition to PA utilization in the present paper one such spontaneous mutant was isolated, physiologically and biochemically characterized and compared with the parent strain. The results suggest that the regulation pattern is changed. Using 2,4-D, MCPA, 2MPA and the corresponding phenols as growth substrates, the regulation of different pathways for the degradation of variously substituted phenoxyacetic acids and phenols was studied.

Materials and methods

Bacterial strain and growth conditions

Alcaligenes eutrophus JMP 134 was isolated on the basis of being able to grow with 2,4-dichlorophenoxyacetic acid (2,4-D) as sole source of carbon and energy (Don and Pemberton 1981). For growth in batch culture, the mineral medium as described by Dorn et al. (1974) contained 5 mM of the respective substrate when grown on fructose or phenoxyacetic acids and 2 mM when grown on phenol or 2-methylphenol. Cells were grown in 100, 1000 or 3000 ml

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fluted Erlenmeyer flasks containing 10, 100 or 500 ml of medium, respectively. The flasks were incubated at 30°C on a rotary shaker at 150 rpm and growth was monitored photometrically by measuring the turbidity at 546 nm. Solid media were prepared by the addition of 1.5% (wt/vol) agar No. 1 (Oxoid) to the mineral medium. The concentration of the carbon source in agar plates was 2 mM. Stock cultures were maintained on 2,4-D-containing agar plates. Continuous cultures were grown at 30°C in 250 ml chemostats as previously described (Knackmuss and Hellwig 1978).

Induction experiments

Cells were grown in 500 ml fluted Erlenmeyer flasks containing 50 ml medium and fructose as carbon source. During late exponential growth phase, this culture was transferred into an 3000 ml fluted Erlenmeyer flask, containing 500 ml of medium, supplemented with fructose (5 mM) and 2 mM of 2,4-D or phenoxyacetic acid (PA). Cells were harvested after 15 h of incubation at 30°C on a rotary shaker at 150 rpm.

Preparation of cell extracts

Cells were harvested during late exponential growth and suspended in phosphate-acetone buffer (100 mM, pH 7.5) (Nozaki 1970) for determination of catechol 2,3-dioxygenase (C230) or 2-hydroxy-6-oxohexa-2,4-dienoate hydrolase (2-hydroxymuconic semialdehyde hydrolase, HMSH) activities and in Tris-hydrochloride buffer (100 mM, pH 7.5) (Dorn and Knackmuss 1978) for the other enzymes tested. The cell suspensions were disrupted using a French-press (Aminco, Silver Spring, MD, USA) and the cell debris removed by centrifugation at $100,000 \times g$ for 1 h at 4°C. Cell extracts were used the same day.

Enzyme assays and activities with whole cells

Enzyme assays and measurement of activities with whole cells were carried out as reported by Pieper et al. (1988).

Analytical methods

The concentrations of substituted phenoxyacetic acids and phenols were determined by high pressure liquid chromatography as previously described (Pieper et al. 1988).

Chemicals

Chemicals were those reported by Pieper et al. (1988). 2,4-Dichlorophenol was obtained from Aldrich Chemie, Steinheim, FRG.

Results

Growth on phenol

Phenol is a growth substrate for *Alcaligenes eutrophus* JMP 134 exhibiting uniform growth after 5 days of incubation on solid medium. In phenol-grown cells substantial activities of catechol 1,2-dioxygenase type I were synthesized (Table 1). This enzyme exhibited only very poor activities against chlorocatechols (Pieper et al. 1988). Muconate cycloisomerase and 4-carboxymethylbut-3-en-4-olide hy-

drolase as well as catechol 2,3-dioxygenase and 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase were also induced, demonstrating the simultaneous presence of both meta- and ortho-cleavage pathway activities. In contrast, activities of enzymes of the "chloroaromatic" pathway [i.e. 2,4-D monooxygenase, chlorophenol hydroxylase (CPH), catechol, 1,2-dioxygenase type II (C120 II), chloromuconate cycloisomerase (CMC), dienelactone hydrolase (DLH) and maleylacetate reductase (MAR)] were not induced.

Phenoxyacetic acids as a potential growth substrate

2,4-D-grown cells cooxidized PA (2 mM) with transient accumulation of phenol. Although 2,4-D monooxygenase exhibits substantial activity against PA and intermediate phenol supports growth, phenoxyacetic acid (PA) is not a growth substrate for *Alcaligenes eutrophus* JMP 134 (Pemberton et al. 1979). As shown in Table 2, the activity of 2,4-D monooxygenase induced by PA was much lower than the activity induced by 2,4-D being approximately the same as in uninduced cells. In contrast, catechol 1,2-dioxygenase I, a cycloisomerase active against muconate and a high level of 4-carboxymethylbut-2-en-4-olide hydrolase were induced.

When JMP 134 was plated on PA containing solid medium spontaneous mutants were observed which were able to use PA as sole source of carbon and energy. Mutant colonies (at least 1 mm in diameter after 7 days of incubation) appeared at a frequency of 10^{-4}. From one of these colonies the derivative strain *Alcaligenes eutrophus* JMP 134-1 was selected for further study.

Constitutive expression of enzymes of the "chloroaromatic pathway" in JMP 134-1

Derivative strain JMP 134-1, isolated for its ability to grow on PA, was analyzed for enzyme expression when grown on fructose. Compared with the parent strain, significant higher levels (5 – 70-fold) of the enzymes 2,4-D monooxygenase, CPH, C120 II, CMC, DLH and MAR were found. This indicates constitutive expression of these enzymes (Table 3). The substrate specifity of 2,4-D monoxygenase in fructose-grown cells of JMP 134-1 (Table 4) was found to be the same as those found in 2,4-D-grown cells of the parent strain (Pieper et al. 1988). With the exception of 2,3- and 3,4-dichlorophenoxyacetic acid, all phenoxyacetic acids tested were shown to be metabolized at rates exceeding 50% of that found for 2,4-D.

Enzyme activities of JMP 134-1 during growth on PA, 2,4-D or phenol

Incubation of fructose-grown cells ($OD_{546nm} = 10$) of JMP 134-1 with PA (2 mM) led to quantitative accumulation of phenol which after an induction period of about 3 h was degraded further. This is consistent with the observation that CPH, constitutively expressed by JMP 134-1, exhibits no activity against phenol (Liu and Chapman 1984; Pieper et al. 1988). In addition to CPH, PA-grown cells of JMP 134-1 induced a further phenol hydroxylating activity. Enzymes of the 3-oxoadipate pathway were also induced, but no enzymes of the meta-cleavage pathway (Table 3).

Although considerable activities of the enzymes of the "chloroaromatic pathway" were found in fructose-grown

Table 1. Specific activities of catabolic enzymes from phenol or 2-methylphenol grown cells of *Alcaligenes eutrophus* JMP 134

Enzyme activity	Assay substrate	sp. act. (U/g protein) after growth with[a]		
		Phenol	2-Methylphenol	Fructose
2,4-D monooxygenase	2,4-D	7	8	6
Phenol hydroxylase	2,4-Dichlorophenol	8	16	12
	Phenol	145	130	<5
	2-Methylphenol	100	95	<5
Catechol 2,3-dioxygenase	Catechol	450	430	<1
2-Hydroxymuconic semialdehyde hydrolase	2-Hydroxy-6-oxohepta-2,4-dienoate	190	190	<10
Catechol 1,2-dioxygenase	Catechol	290	45	6
	3-Chlorocatechol	6	3	6
Muconate cycloisomerase	*cis,cis*-Muconate	45	9	<5
	2,4-Dichloro-*cis,cis*-muconate[b]	<5	<5	<5
4-Carboxymethylbut-3-en-4-olide hydrolase	4-Carboxymethyl-3-en-4-olide	380	20	30
4-Carboxymethylenebut-2-en-4-olide hydrolase	*trans*-4-Carboxymethylenebut-2-en-4-olide	50	45	55
Maleylacetate reductase	Maleylacetate	15	15	15

[a] Cells were harvested during exponential growth phase. Enzyme activities of 2,4-D monooxygenase and phenol hydroxylases were determined with whole cells. The specific oxygen uptake with phenols was divided by two, to get comparable activities. All other activities were determined in cell extracts

[b] 3,5-Dichlorocatechol was cleaved by partially purified catechol 1,2-dioxygenase of the type II and the accumulating product was used as assay substrate as described in the Materials and methods section

Table 2. Specific activities of catabolic enzymes from cells of *Alcaligenes eutrophus* JMP 134 induced with 2,4-dichlorophenoxyacetic acid (2,4-D) or phenoxyacetic acid PA)

Enzyme activity	Assay substrate	sp. act. (U/g protein) after induction with[a]		
		2,4-D	PA	Fructose
2,4-D monooxygenase	2,4-D	32	9	6
Phenol hydroxylase	2,4-Dichlorophenol	130	17	12
	Phenol	<5	<5	<5
	2-Methylphenol	<5	<5	<5
Catechol 2,3-dioxygenase	Catechol	<1	<1	<1
2-Hydroxymuconic semialdehyde hydrolase	2-Hydroxy-6-oxohepta-2,4-dienoate	<10	<10	<10
Catechol 1,2-dioxygenase	Catechol	310	150	6
	3-Chlorocatechol	190	15	10
Muconate cycloisomerase	*cis,cis*-Muconate	<5	45	<5
	2,4-Dichloro-*cis,cis*-muconate[b]	240	<5	<5
4-Carboxymethylbut-3-en-4-olide hydrolase	4-Carboxymethylbut-3-en-4-olide	60	1300	30
4-Carboxymethylenebut-2-en-4-olide hydrolase	*trans*-4-Carboxymethylenebut-2-en-4-olide	760	100	55
Maleylacetate reductase reductase	Maleylacetate	500	120	15

[a] Cells were harvested after 15 h of induction. See Table 1, footnote a, for further details

[b] See Table 1, footnote b, for details of the enzymatic test

cells of JMP 134-1, even higher levels of 2,4-D monooxygenase, CPH and MAR were induced during growth on PA (Table 3). The same was true for 2,4-D and phenol-grown cells. Activities of C120 II, CMC and DLH in contrast were always approximately the same as those in fructose-grown cells. No significant differences were found in enzyme levels of 2,4-D-grown cells of the mutant compared with the parent strain (Pieper et al. 1988). Like JMP 134, JMP 134-1 exhibited ortho-cleavage as well as meta-cleavage activities when grown on phenol.

Growth of JMP 134 and JMP 134-1 with 2,4-dichlorophenol, 4-chloro-2-methylphenol and 2-methylphenol

Since the corresponding phenols were found to be intermediates of the degradation of 2,4-D, MCPA and 2MPA by JMP 134 (Pieper et al. 1988), these compounds were tested as

potential growth substrates and inducers of peripheral as well as ortho- and meta-cleavage pathway enzymes. Because of the high toxicity of 2,4-dichlorophenol and 4-chloro-2-methylphenol growth with these compounds was carried out in continuous culture.

Cells of JMP 134 pregrown in batch culture containing 5 mM 2,4-D or 5 mM MCPA were transferred to 250 ml chemostats. Fresh medium was pumped into the culture at dilution rates of $0.085 h^{-1}$ or $0.06 h^{-1}$, respectively. Phenoxyacetic acids as growth substrate were gradually replaced by the corresponding phenols over a period of about 3 weeks, so that the total concentration of aromatic substrate was always 5 mM. Up to a relative concentration of 0.1/4.9 (mM phenoxyacetic acid/mM phenol) the cultures remained stable as indicated by its optical density ($OD_{546nm} > 0.5$). Below this threshold value both the 2,4-D and the MCPA culture were washed out indicating that both 2,4-dichloro-

Table 3. Specific activities of catabolic enzymes from fructose, phenoxyacetic acid (PA), 2,4-dichlorophenoxyacetic acid (2,4-D) or phenol grown cells of *Alcaligenes eutrophus* JMP 134-1

Enzyme activity	Assday substrate	sp. act. (U/g protein) after growth with[a]			
		Fructose	PA	2,4-D	Phenol
2,4-D monooxygenase	2,4-D	40	75	110	70
Phenol hydroxylase	2,4-Dichlorophenol	55	160	210	140
	Phenol	<5	150	110	150
	2-Methylphenol	<5	55	45	160
Catechol 2,3-dioxygenase	Catechol	<1	7	<1	290
2-Hydroxymuconic semialdehyde hydrolase	2-Hydroxy-6-oxohepta-2,4-dienoate	<10	<10	<10	180
Catechol 1,2-dioxygenase	Catechol	360	590	740	560
	3-Chlorocatechol	440	540	520	390
Muconate cycloisomerase	*cis,cis*-Muconate	<5	580	16	240
	2,4-Dichloro-*cis,cis*-muconate[b]	315	300	520	295
4-Carboxymethylbut-3-en-4-olide hydrolase	4-Carboxymethylbut-3-en-4-olide	30	1740	60	930
4-Carboxymethylenebut-2-en-4-olide hydrolase	*trans*-4-Carboxymethylenebut-2-en-4-olide	2130	2090	3030	2010
Maleylacetate reductase	Maleylacetate	110	380	500	290

[a] Cells were harvested during exponential growth phase. See Table 1, footnote a, for further details
[b] See Table 1, footnote b, for details of the enzymatic test

Table 4. Relative rates of conversion of various substituted phenoxyacetic acids by freshly harvested cells of *Alcaligenes eutrophus* JMP 134-1 grown on fructose

Assay substrate	Relative rate of conversion[a]
2,4-Dichlorophenoxyacetic acid	100 (40)
4-Chloro-2-methylphenoxyacetic acid	70
Phenoxyacetic acid	75
2-Chlorophenoxyacetic acid	80
4-Chlorophenoxyacetic acid	75
2-Methylphenoxyacetic acid	50
4-Methylphenoxyacetic acid	110
2-Chloro-4-methylphenoxyacetic acid	110
2,4-Dimethylphenoxyacetic acid	85
2,3-Dichlorophenoxyacetic acid	10
3,4-Dichlorophenoxyacetic acid	5

[a] Rates are expressed as percentages of that for 2,4-dichlorophenoxyacetic acid (= 100%). The specific activity after growth on fructose, given in micromoles substrate per min per gram protein, appear in parentheses

and 4-chloro-2-methylphenol were no growth substrates for JMP 134 when applied as single compounds. In equivalent experiments with JMP 134-1, no washing out was observed, even after complete replacement of the phenoxyacetic acids by the corresponding phenols. The mutant strain JMP 134-1 clearly utilized 2,4-dichlorophenol or 4-chloro-2-methylphenol as sole sources of carbon and energy.

The meta-cleavage pathway enzymes were not induced neither in JMP 134 nor in JMP 134-1 when grown in continuous culture with these compounds. This was demonstrated by the absence of catechol 2,3-dioxygenase (C230) and hydroxymuconic semialdehyde hydrolase (HMSH) activity in cell free extracts. In contrast, 2-methylphenol served as sole growth substrate of the parent strain JMP 134 and the enzymes C230 and HMSH were induced at high levels (Table 1). Although enzymes of the chlorocatechol ortho-cleavage pathway were not induced, low activity of catechol 1,2-dioxygenase I indicated, that some methylcatechol might also be metabolized via an ortho-cleavage pathway.

Discussion

The cleavage of the ether bond of phenoxyacetic acid (PA), 4-chloro-2-methylphenoxyacetic acid (MCPA) and 2-methylphenoxyacetic acid (2MPA) by *Alcaligenes eutrophus* JMP 134 is mediated by the same monooxygenase which also functions in the catabolism of 2,4-dichlorophenoxyacetic acid (2,4-D) (Fig. 1). Since phenol unlike PA is a growth substrate for JMP 134, the crucial step of PA-degradation might be the induction of this first catabolic enzyme. That permeation is not critical in JMP 134 could be shown by the induction of enzymes of the 3-oxoadipate pathway such as catechol 1,2-dioxygenase I, muconate cycloisomerase and 4-carboxymethylbut-3-en-4-olide hydrolase when incubated with PA. 2,4-D monooxygenase, however, is one of the enzymes of the "chloroaromatic pathway" which were not induced in presence of PA. In contrast to the wild type organism, fructose-grown cells of the PA-utilizing mutant strain *Alcaligenes eutrophus* JMP 134-1 showed constitutive levels of enzymes of the "chloroaromatic pathway" including 2,4-D monooxygenase. The high frequency of mutation generating the PA⁺-phenotype, which has also been observed by Pemberton et al. (1979), indicated that a single mutational event is involved.

The genes coding for the enzymes 2,4-D monooxygenase, chlorophenol hydroxylase (CPH), catechol 1,2-dioxygenase II (C120 II), chloromuconate cycloisomerase (CMC) and dienelactone hydrolase (DLH) have been localized on the plasmid pJP4 (Don et al. 1985; Streber et al. 1987) and found to be organized in at least three different transcriptional units. Only the gene coding for maleylacetate reductase (MAR) has not yet been localized and is probably encoded on a fourth transcriptional unit. Although growth on PA requires only a single enzyme, namely 2,4-D monooxygenase, to be produced constitutively, obviously five other enzymes encoded on different transcriptional units were also synthesized constitutively by the mutant selected for growth on PA (Table 3). Probably a common regulatory protein regulates the expression of the different transcriptional units as shown in phenol and naphthalene catabolism (Wigmore et al. 1977; Bayly et al. 1977; Yen and Gunsalus 1982).
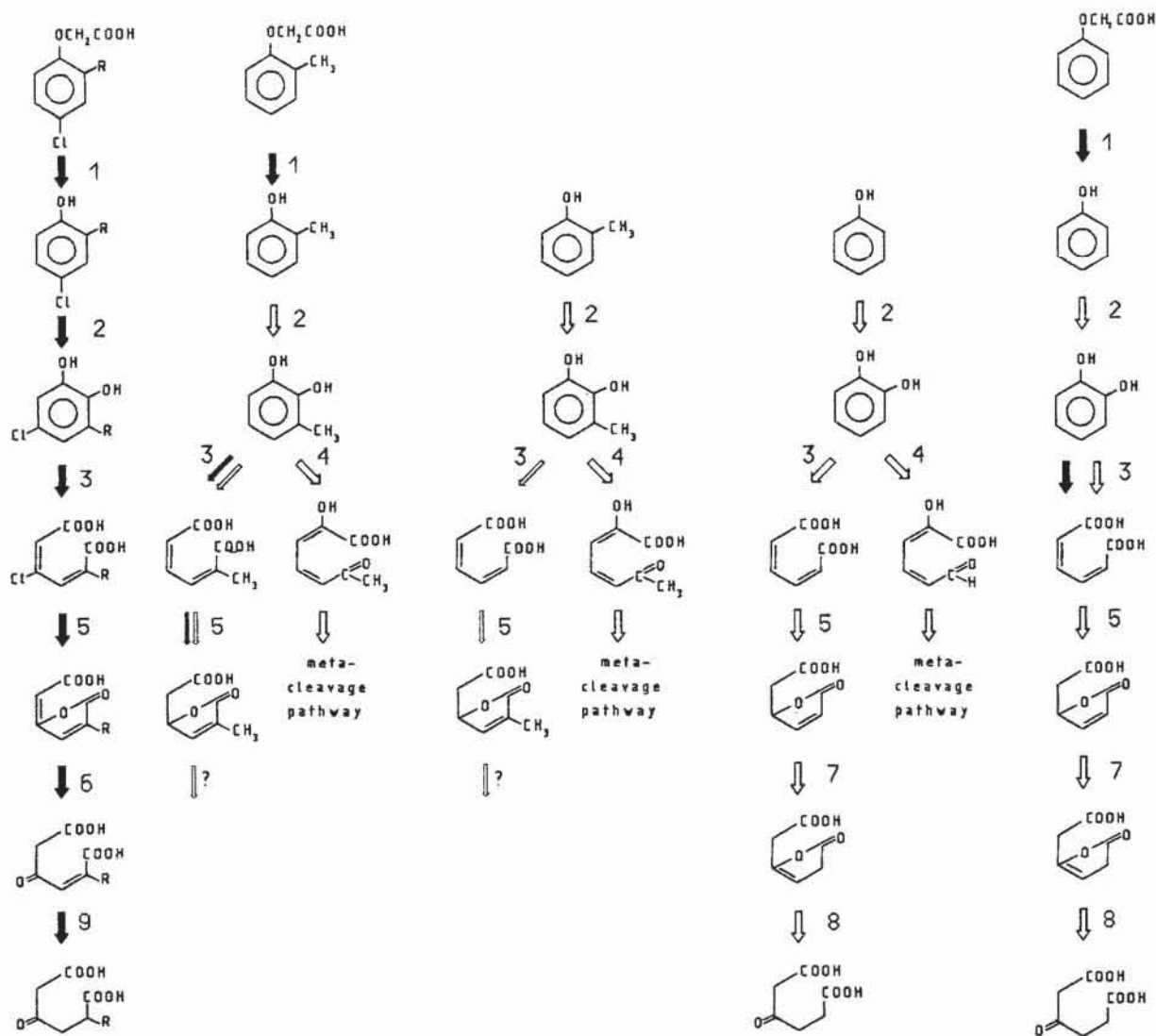
Fig. 1. Proposed pathways for the utilization of 2,4-dichlorophenoxyacetic acid (R = Cl), 4-chloro-2-methylphenoxyacetic acid (R = CH$_3$), 2-methylphenoxyacetic acid, 2-methylphenol or phenol in *Alcaligenes eutrophus* JMP 134 and of phenoxyacetic acid in *Alcaligenes eutrophus* JMP 134-1. *Solid arrows* indicate enzymes primarily functioning in the "chloroaromatic pathway". All other enzymes, indicated by *hollow arrows*, originate from aromatic or methylaromatic pathways. If these activities were only slightly induced during growth with the above substrates (less than 20% of inducible activity), they were marked with *slim arrows*. The enzymes are as follows: *1* 2,4-D monooxygenase; *2* phenol hydroxylating activities (chlorophenol hydroxylase, phenol hydroxylase, methylphenol hydroxylase); *3* catechol 1,2-dioxygenating activities (catechol 1,2-dioxygenase I, II); *4* catechol 2,3-dioxygenase; *5* cis,cis-muconate cycloisomerizing activities (chloromuconate cycloisomerase, *cis,cis*-muconate cycloisomerase); *6* dienelactone hydrolase; *7* 4-carboxymethylbut-2-en-4-olide isomerase; *8* 4-carboxy-methylbut-3-en-4-olide hydrolase; *9* maleylacetate reductase. Gratuitously induced enzymes are not shown

Chemostat cultures showed, that strain JMP 134-1 could grow on either 2,4-dichlorophenol or 4-chloro-2-methyl-phenol, whereas strain JMP 134 could not. Clearly, the induction potential of 2,4-dichloro- as well as 4-chloro-2-methylphenol is insufficient for growth. This identifies 2,4-D and MCPA to be the effectors of at least CPH. Assuming a single regulatory protein, which interacts with the operon coding for CPH and also with other operons coding for enzymes of the "chloroaromatic pathway", it is likely that 2,4-D and MCPA are effectors for the synthesis of all these enzymes. Apparently, also 2MPA, unlike 2-methylphenol induces all enzymes of the "chloroaromatic pathway". Recently, Bruhn et al. (1988) observed induction of the chlorocatechol degrading enzymes in 4-chloro-2-nitro-phenol degrading hybrid organisms with JMP 134 acting as

donor of this catabolic sequence. To explain this phenomenon, structurally dissimilar compounds like phenoxyacetic acids on one hand and catechols or later metabolites on the other must be inducers. A similar observation has been made for the induction of catechol 1,2-dioxygenase in *Acineto-bacter calcoaceticus* (Neidle and Ornston 1987).

Present investigations demonstrate that constitutive levels of C120 II, CMC and DLH in JMP 134-1 were approximately the same as those in 2,4-D-grown cells of the parent strain. They could not be superinduced by 2,4-D (Table 3) and can therefore be termed strictly constitutive (Collins et al. 1965). In contrast, activity levels of 2,4-D monooxygenase, CPH and MAR in fructose-grown cells of JMP 134-1 were only semiconstitutive (Collins et al. 1965). As confirmed by repeated measurements activities of these

enzymes in PA- or phenol-grown cells of JMP 134-1 were significantly higher than in fructose grown cells (Table 3). Under the same conditions these compounds were inefficient as inducers in the parent strain. Obviously fructose (as well as succinate or acetate, data not shown) seems to inhibit the expression of these genes. Although all enzymes of the chloroaromatic pathway seem to be regulated by a common effector, different regulatory mechanisms must exist for 2,4-D monooxygenase and CPH on the one hand and C120 II, CMC and DLH on the other. Current investigations will show whether or not DNA-rearrangements on the plasmid pJP4 (Ghosal et al. 1985) are responsible for the PA-degrading phenotype.

As observed with other microorganisms (Feist and Hegeman 1969; Hughes and Bayly 1983; Sala-Trepat et al. 1972) phenol-grown cells of either parent strain JMP 134 or mutant strain JMP 134-1 as well as 2-methylphenol-grown cells of the parent strain induced high activities of enzymes of the meta-cleavage pathway (Fig. 1). This catabolic route has been shown to interfere with bacterial growth on chloroaromatics (Latorre et al. 1984; Reineke and Knackmuss 1980; Reineke et al. 1982; Rubio et al. 1986). In JMP 134 induction of meta-cleavage pathway during growth on chloroaromatics is prevented because chlorosubstituted phenols such as 2,4-dichloro- or 4-chloro-2-methylphenol exhibited no induction capacity.

Although unsubstituted phenol was shown to be inducer of the enzymes of the meta-cleavage pathway, strain JMP 134 induced with PA or strain JMP 134-1 growing with this substrate exhibited negligible activity of catechol 2,3-dioxygenase (Fig. 1). This indicates a second mechanism for preventing meta-cleavage activity. Apparently, in JMP 134-1 methylphenol hydroxylase plus catechol 2,3-dioxygenase are only induced if high concentrations of phenol are present in the growth medium.

As shown by the present results JMP 134 effectively regulates the utilization of 2,4-D by a modified ortho-cleavage pathway or 2MPA mainly through a meta-cleavage route. In contrast during growth with mixture of these substrates both pathways are induced and catechol 2,3-dioxygenase activity must be suppressed by a wasteful suicide inactivation mechanism (Bartels et al. 1984; Pieper 1986). Knackmuss (1984) developed a strategy to achieve simultaneous degradation of methyl- and chlorosubstituted aromatics via a bifunctional ortho-cleavage pathway. Following this approach, Rojo et al. (1987) succeeded to construct in vitro a derivative of *Pseudomonas* sp. B 13. By recruitment of a gene from the *Alcaligenes eutrophus* JMP 134 chromosome encoding "4-methyl-2-enelactone isomerase" being responsible for the conversion of 4-carboxymethyl-4-methyl- to 4-carboxymethyl-3-methylbut-2-ene-4-olide (Pieper et al. 1985) this strain can assimilate 4-methyl- and 4-chlorocatechol simultaneously.

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