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Modified *ortho*-cleavage pathway in *Alcaligenes eutrophus* JMP134 for the degradation of 4-methylcatechol

(Biodegradation of 4-carboxymethyl-methylbut-2-en-1,4-olides, methylphenoxyacetates; *Alcaligenes eutrophus* JMP134; *ortho*-pathway; simultaneous degradation of chloro- and methylaromatics)

D.H. Pieper, K.-H. Engesser, R.H. Don *, K.N. Timmis * and H.-J. Knackmuss

Bergische Universität -GH, Chemische Mikrobiologie, Gausstrasse 20, D-5600 Wuppertal-1, F.R.G. and * Université de Genève, Faculté de Médecine, Département de Biochimie Médicale, Centre Médical Universitaire, 9, Avenue de Champel, 1211 Geneva 4, Switzerland.

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1. SUMMARY

2,4-Dichlorophenoxyacetate-grown cells of *Alcaligenes eutrophus* JMP134 [1] metabolized 4-methylphenoxyacetate via a modified *ortho*-cleavage pathway. 4-Carboxymethyl-4-methylbut-2-en-1,4-olide (4-methyl-2-enelactone), 4-carboxymethyl-3-methylbut-2-en-1,4-olide (3-methyl-2-enelactone) and 4-methyl-3-oxoadipate, were identified as intermediates.

2. INTRODUCTION

Microbial degradation of methyl-substituted aromatics normally proceeds via *meta*-cleavage pathways with methylcatechols as key intermediates [2-4]. A problem of practical concern during waste water treatment is the degradation of methylaromatics in the presence of chloroaromatics. Because of biochemical and genetic inactivation of *meta*-cleavage activities provoked by 3-chlorocatechols, chloroaromatics-degrading populations lose their ability to utilize methylaromatics [5]. As a consequence of acclimatisation to chloroaromatics, methylcatechols are subject to unproductive *ortho*-cleavage with accumulation of methyl-substituted 4-carboxymethyl-but-2-en-1,4-

olides as dead-end metabolites [6,7].

In contrast, a *Nocardia* strain was reported to metabolize 4-methylbenzoate via an unusual *ortho*-cleavage pathway [8]. 4-Carboxymethyl-3-methylbut-2-en-1,4-olide (3-methyl-2-enelactone) was postulated as the primary cycloisomerisation product of 3-methyl-*cis,cis*-muconic acid. This unusual cycloisomerisation mechanism would circumvent the problem of isomerisation of but-2-en-1,4-olides to but-3-en-1,4-olides, which is an essential feature of the classical *ortho*-pathway [9]. This isomerisation step is obviously impossible in the case of the 4-methyl-2-enelactone.

A. eutrophus JMP134 is known to degrade both 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA). The possibility that it is able to degrade methylaromatics via an *ortho* pathway was thus worthy of investigation, and our preliminary findings are presented here.

3. MATERIALS AND METHODS

3.1. Bacterial strains

A. eutrophus JMP134 was isolated on the basis of being able to grow with 2,4-D as sole source of carbon and energy [1]. Most enzymes of the 2,4-D

catabolic pathway are encoded on a 75 kb plasmid, pJP4, present in this organism [10,11]. *A. eutrophus* JMP222 is a derivative of JMP134 that has lost pJP4, and is therefore unable to catabolize 2,4-D. Both JMP134 and JMP222 are able to grow on 4-methyl-2-enelactone as sole carbon source (this paper). Strain *A. eutrophus* RD553 is a mutant derivative of JMP222 obtained by transposon mutagenesis with transposon Tn5 using the suicide vector pLG221 [10], which is unable to utilize 4-methyl-2-enelactone as sole carbon source.

3.2. Growth conditions

For accumulation of metabolites, resting cells of *A. eutrophus* JMP134 were used. These were obtained by growth in a mineral medium [12] with 2,4-dichlorophenoxyacetate (2,4-D) or 4-methyl-2-enelactone (5 mM each) as sole carbon source. Cells were harvested, resuspended in phosphate buffer (A_{546nm} approx. 5) and incubated at 30° with the respective substrate (2 mM).

Cells of strain RD553 were pregrown in a mineral medium containing 5 mM fructose and 50 µg/ml kanamycin. Conversion of 4-methyl-2-enelactone (3 mM) was carried out by incubation in the presence of 2 mM fructose.

3.3. Identification and isolation of metabolites

The concentration of 4-methyl-2-enelactone and 3-methyl-2-enelactone in the culture supernatant fluid was determined by HPLC analysis at 210 nm (Column SC with Lichrospher 100 spheric RP8, Bischoff, Leonberg, F.R.G.) and by comparison with authentic compounds [6]. The solvent system contained 80 ml MeOH and 1 g H₃PO₄ per l. Under these conditions, the relative retention volume compared with catechol (3 ml) was 0.6 for 4-methyl- and 0.56 for 3-methyl-2-enelactone. Ultraviolet spectra were determined by use of a Shimadzu UV 240 spectrophotometer. Mass spectra were recorded on a MAT 311A spectrometer (Varian, Bremen, F.R.G.).

NMR spectra were recorded on a FT 80 spectrometer in the case of 3-methyl-2-enelactone and on a XL 200 spectrometer (Varian, Bremen, F.R.G.) for 4-methyl-3-oxoadipic acid dimethylester with tetramethylsilane as internal standard and CDCl₃ as solvent.

For isolation and purification of 4-methyl-3-oxoadipic acid and its dimethylester, the cell-free culture fluid was acidified to pH 3 with H₃PO₄ and extracted by ethylacetate. The extracted metabolite was stabilized by methylation with diazomethane [13]. The dimethylester of 4-methyl-3-oxoadipic acid was purified by preparative thin-layer-chromatography on silica gel 60 mesh PF 254 (Merck, Darmstadt, F.R.G.) with 70% diisopropylether, 25% hexane and 5% ethylacetate as the mobile solvent. Bands were detected under UV-light (254 nm).

4. RESULTS AND DISCUSSION

2,4-D-grown cells of *A. eutrophus* JMP134 [1] readily converted 4-methylphenoxyacetate (4MPA) almost quantitatively into 4-methyl-2-enelactone. Unlike *Pseudomonas* sp. B13 [6], strain JMP134 harbors the capability of further metabolism of 4-methyl-2-enelactone. Thus, after a short induction period, the metabolite disappeared from the culture fluid, with concomitant excretion of another metabolite exhibiting a slightly different retention volume on the reversed-phase column.

During further incubation this metabolite also disappeared. Cells of *A. eutrophus* JMP134, pregrown with 4-methyl-2-enelactone, showed the same phenomenon of temporary accumulation of the new metabolite, when incubated with 4-methyl-2-enelactone.

By use of cell-free extracts from 4-methyl-2-enelactone-grown cells, 4-methyl-2-enelactone was quantitatively converted into the new metabolite. Further degradation was not observed *in vitro*. In comparison to 4-methyl-2-enelactone, the UV-spectrum of the new compound showed a slight increase in absorption and shift of λ_{max} from 212 nm to 215 nm. By analogy with the postulated *ortho*-cleavage pathway of methylbenzoate in *Nocardia* [8], this metabolite was assumed to be 4-carboxymethyl-3-methylbut-2-en-1,4-olide (3-methyl-2-enelactone). In order to verify this hypothesis, the 4-methyl-2-enelactone was chemically converted into 3-methyl-2-enelactone following the procedure of Catelani [7].

Using slightly alkaline conditions (pH 8.5) and

elevated temperature (70°C), 4-methyl-2-enelactone was transformed quantitatively into a compound, which was undistinguishable from the biologically produced metabolite of 4-methyl-2-enelactone. The identity of both compounds was confirmed by HPLC analysis as well as by UV spectrometry. Furthermore, the chemically generated compound was readily degraded by whole cells of *A. eutrophus* JMP134, pregrown on 4-methyl-2-enelactone. Its structure was shown by mass spectrometry to be 4-carboxymethyl-3-methylbut-2-en-1,4-olide (3-methyl-2-enelactone, Table 1). The very intense molecular ion at m/z : 156 (M^+) was identified by high resolution technique. The fragmentation pattern observed was essentially the same as that described in the literature [7,8].

The $^1\text{H-NMR}$ spectrum (Table 2) again coincided well with the reference data [7,8].

Since 3-methyl-2-enelactone was not further transformed by cell free extracts the fate of this metabolite was investigated by means of transposon mutants of *A. eutrophus* JMP222, which were defective in this pathway and had therefore lost the ability to grow with 4-methyl-2-enelactone as sole carbon source.

After induction of one of these mutants, strain RD553, with 4-methyl-2-enelactone, a metabolite was detected after the inducer had been completely metabolized. The metabolite was extracted from the acidified culture fluid and purified by thin layer chromatography. The mass spectrum of the dimethyl derivative revealed a molecular ion of

Table 1

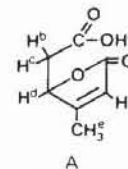
Mass spectrum of 4-carboxymethyl-3-methylbut-2-en-1,4-olide (3-methyl-2-enelactone)

m/z	Intensity (% of base peak)	Assignment
156	7.9	M^+
138	12.4	$M^+ - \text{H}_2\text{O}$
111	11.6	$M^+ - \text{COOH}$
110	82.8	$M^+ - \text{HCOOH}$
97	51.1	$M^+ - \text{CH}_2\text{COOH}$
96	22.4	$(M^+ - \text{CH}_2\text{COOH}) - \text{H}$
69	100.0	$(M^+ - \text{CH}_2\text{COOH}) - \text{CO}$
68	37.5	$[(M^+ - \text{CH}_2\text{COOH}) - \text{CO}] - \text{H}$

Table 2

$^1\text{H-NMR}$ -spectrum of 4-carboxymethyl-3-methylbut-2-en-1,4-olide (3-methyl-2-enelactone)

Proton	δ (ppm) ^a	Description ^b
a	8.71	1H, s
b	2.57	1H, q, $J_{bc} = 16$, $J_{bd} = 8$
c	2.85	1H, q, $J_{bc} = 16$, $J_{cd} = 4$
d	5.17	1H, m, $J_{bd} + J_{cd} = 12$
e	2.07	3H, m, $J_{ef} = 1$
f	5.82	1H, m, $J_{ef} = 1$



^a Chemical shifts (δ) are given as ppm downfield from tetramethylsilane in CDCl_3 .

^b Singlet, quartet and multiplet are abbreviated to s, q and m respectively. Coupling constants (J) are given in Hz.

m/z : 202(M^+), indicating a methyloxadipic acid dimethylester. High resolution technique revealed the empirical formula $\text{C}_9\text{H}_{14}\text{O}_5$. The mass spectrum (Table 3) showed a fragmentation pattern typical for an ester of a dicarboxylic acid (m/z : 171, 170, 143, 139, 59). The presence of a central carboxylic function is demonstrated by the well-known α -fragmentation (m/z : 129, 101).

The integral of the resonance lines of the $^1\text{H-NMR}$ spectrum (Table 4) corresponds to the expected 14 protons. The 2 singlets of $\delta = 3.67$ and 3.76 ppm are due to 2 ester methyl groups (protons h and a). The protons b and c are represented by 2 doublets centered at $\delta = 3.59$ and 3.64 ppm, respectively. The intensities of both doubled constituting signals are extremely different, due to the

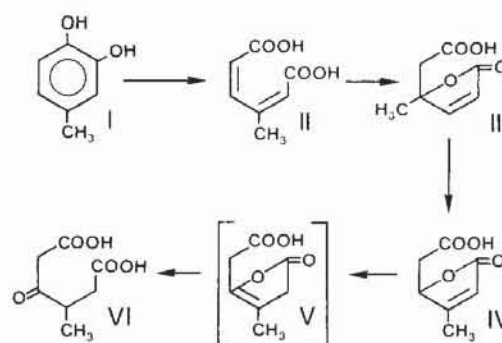


Fig. 1. Pathway for the degradation of 4-methylcatechol in *A. eutrophus* JMP134. I, 4-methylcatechol; II, 3-methyl-cis,cis-muconic acid; III, 4-methyl-2-enelactone; IV, 3-methyl-2-enelactone; VI, 4-methyl-3-oxoadipic acid. Substance V, 4-carboxy-methyl-3-methylbut-3-en-1,4-olide, is postulated as an intermediate.

Table 3

Mass spectrum of 4-methyl-3-oxoadipic acid dimethylester

<i>m/z</i>	Intensity (% of base peak)	Assignment
202	1.3	M ⁺
171	10.7	M ⁺ -OCH ₃
170	25.6	M ⁺ -HOCH ₃
143	15.3	(M ⁺ -OCH ₃)-CO
139	8.4	(M ⁺ -HOCH ₃)-OCH ₃
129	33.0	M ⁺ -CH ₂ COOCH ₃
102	42.0	(M ⁺ -CH(CH ₃)-CH ₂ -COOCH ₃)+H
101	79.2	M ⁺ -CH(CH ₃)-CH ₂ -COOCH ₃)
69	33.4	[(M ⁺ -OCH ₃)-CO]-CH ₂ =C(OH)OCH ₃
59	100.0	M ⁺ -CH ₂ -CO-CH(CH ₃)-CH ₂ -COOCH ₃ and M ⁺ -CH ₂ -CH(CH ₃)-CO-CH ₂ -COOCH ₃

very similar chemical shift of proton b and c. Protons g and f are centered at $\delta = 2.37$ and 2.82 ppm, respectively (the difference in the chemical shift is caused by the proximity to the asymmetric carbon atom) and are split further into 2 quartets due to the vicinal coupling of proton d. The protons of the methyl group attached to the asymmetric carbon atom (proton e) resonate at $\delta = 1.19$ ppm. Proton d shows a most complicated coupling pattern, consisting of fourteen signals (for detailed coupling analysis, see Table 4). These data are consistent with the proposed structure of 4-methyl-3-oxoadipic acid dimethylester. A modified *ortho*-cleavage pathway for the degradation of 4-

methylcatechol in *A. eutrophus* JMP134 is proposed in Fig. 1.

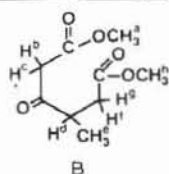
If 4-methyl-3-oxoadipate is metabolized in accordance with the classical 3-oxoadipate pathway methylsuccinate must be an intermediate. Preliminary results indicate that *A. eutrophus* JMP134 readily acquires the capability to utilize methylsuccinate by spontaneous mutation.

The present study shows that catabolic activities exist in nature not only for methyl- and chloro-substituted aromatic compounds as sole carbon sources, but also for mixtures of these chemicals, which under certain conditions are incompatible substrates.

Table 4

¹H-NMR-spectrum of 4-methyl-3-oxoadipic acid dimethylester

Proton	δ (ppm) ^a	Description ^b
a	3.76	3H, s
b	3.64	1H, d, $J_{bc} = 14.5$
c	3.59	1H, d, $J_{bc} = 14.5$
d	3.13	1H, m, $J_{de} = 7$, $J_{df} = 5.5$, $J_{dg} = 8.5$
e	1.19	3H, d, $J_{de} = 7$
f	2.82	1H, q, $J_{df} = 5.5$, $J_{fg} = 17$
g	2.37	1H, q, $J_{dg} = 8.5$, $J_{fg} = 17$
h	3.67	3H, s



^a Chemical shifts (δ) are given as ppm downfield from tetramethylsilane in CDCl₃.

^b Singlet, doublet, quartet and multiplet are abbreviated to s, d, q and m respectively. Coupling constants (*J*) are given in Hz.

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