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Degradation of 4-chloro-2-methylphenol by an activated sludge isolate and its taxonomic description

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Abstract

The Gram-negative strain S1, isolated from activated sludge, metabolized 4-chloro-2-methylphenol by an inducible pathway via a modified *ortho*-cleavage route as indicated by a transiently secreted intermediate, identified as 2-methyl-4-carboxymethylenebut-2-en-4-olide by gas chromatography/mass spectrometry. Beside 4-chloro-2-methylphenol only 2,4-dichlorophenol and 4-chlorophenol were totally degraded, without an accumulation of intermediates. The chlorinated phenols tested induced activities of 2,4-dichlorophenol hydroxylase and catechol 1,2-dioxygenase type II. Phenol itself appeared to be degraded more efficiently via a separate, inducible *ortho*-cleavage pathway. The strain was characterized with respect to its physiological and chemotaxonomic properties. The fatty acid profile, the presence of spermidine as main polyamine, and of ubiquinone Q-10 allowed the allocation of the strain into the α-2 subclass of the *Proteobacteria*. *Ochrobactrum anthropi* was indicated by fatty acid analysis as the most similar organism, however, differences in a number of physiological features (e.g. absence of nitrate reduction) and pattern of soluble proteins distinguished strain S1 from this species.

Abbreviations: 4C2MP – 4-chloro-2-methylphenol, 2,4D – 2,4-dichlorophenoxyacetic acid, MCPA – 4-chloro-2-methylphenoxyacetic acid

Introduction

Phenoxyalkanoic acids such as 2,4-dichlorophenoxyacetic acid (2,4 D) and 4-chloro-2-methylphenoxyacetic acid (MCPA) are widely used in agriculture and forestry as herbicides against dicotyledonous weeds. Their production generates waste waters heavily contaminated with the herbicides and related chlorophenols used as precursors of their synthesis. Because even low concentrations of chlorinated aromatics have been reported to inhibit pure bacterial cultures (Tyler & Finn 1974; Pieper et al. 1989; Bestetti et al. 1992), their high toxicity may cause problems during waste water treatment. Consequently, a skilful process control has to be used in the waste water treatment system of a herbicide factory in order to avoid chlorophenol shock loads and to allow bacteria, that actively degrade chlorophenols, to become established in the activated sludge.

Here we describe the isolation of a 4-chloro-2-methylphenol (4C2MP)-mineralizing bacterium from the aeration tank of such a factory. The isolated bacterium was characterized with respect to the degradation pathways involved, substrate specificity, and physiological and chemotaxonomic traits.

Materials and methods

Bacterial strains

The bacterium S1 was isolated from activated sludge. *Ochrobactrum anthropi* strains LMG 3331^T (= CIP 149–70 = NCTC 12168) and LMG 7991 were received from the Culture Collection of the Laboratorium voor Microbiologie, Rijksuniversiteit Gent, Belgium (LMG).

Media and culture conditions

Strain S1 was maintained on nutrient agar I (Berlin-Chemie AG, Germany). For taxonomic characterization, large quantities of non-induced cells were collected from overnight grown cultures in nutrient broth I. The usual growth temperature was 28 to 30 °C. For degradation experiments, a mineral salt solution (Straube et al. 1990) was used with the modification that trace elements were applied at a ten fold higher concentration. Phenolic substrates were added as filter sterilized stock solutions in appropriate amounts to the autoclaved medium. Batch cultivation was carried out in 500 ml flasks filled with 50 or 100 ml medium on a rotary shaker. To induce the degradative enzymes, log-cells were harvested from nutrient broth I and resuspended (0.5 $g \cdot l^{-1}$) in mineral salt solution, supplied with 0.1 $g \cdot l^{-1}$ yeast extract and 0.15 mM of the respective substrate. Immediately after consumption of the respective xenobiotic substance, the cells were centrifuged (10 min, 6000 rpm) and used to study degradation kinetics and formation of intermediates in batch cultures or to prepare crude extracts (experiments given in Figs. 3 and 4 and Tables 2 and 3). Usually, 4C2MP, 2,4-dichlorophenol, 4-chlorophenol and phenol were totally consumed within 2 to 5 h. The interval of exposure to non or very slowly degraded compounds as the trichlorophenols, 2-methylphenol, MCPA, 2- and 3-chlorophenol was also in this range. To confirm the inducibility of the 4C2MP degradation pathway, both log-cells from nutrient broth and 4C2MP-induced cells were harvested, washed with mineral salt solution, resuspended to give a cell density corresponding to 0.5 g dry weight $\cdot l^{-1}$, and incubated with chloramphenicol (20 $mg \cdot l^{-1}$) for 20 min. Then 4C2MP (25 $mg \cdot l^{-1}$) was supplied, and its degradation was followed using the 4-aminoantipyrine method (Lacoste et al. 1959). Continuous cultures were carried out in a Biotec-fermenter (working volume 2.4 l) at 30 °C, at 350 rpm and an airation of 180 $l \cdot min^{-1}$. Fresh medium supplemented with 4C2MP (3.5 $g \cdot l^{-1}$) was added continuously at flow rates between 19 and 55 ml per hour.

Waste water treatment

A phenoxyalkanoic acids-producing chemical company (SYS Schwarzheide) purified its waste water biologically in 3 steps, using an aeration and a nitrification tank and a denitrification system. The herbicidal waste stream, which amounted to 10% of the total waste water entering the activated sludge basin, contained 5 different 4-chloro-2-methylphenol- and 2,4-dichlorophenol-derived phenoxyalkanoic herbicides, the corresponding phenols and also 2,2-dichloropropionic acid (Omnidel) at concentrations of 1.58, 0.66 and 2.3 $g \cdot l^{-1}$ (annual average), respectively (Bloedorn 1991).

Enrichment of 4C2MP-degrading bacteria

Two liters of activated sludge from the aeration tank of the above mentioned waste water treatment system was stored overnight at 4 °C, centrifuged and washed twice with sterile water, resuspended in mineral salt solution and filled into the chemostat vessel. Enrichment of 4C2MP-degrading bacteria was carried out by a combination of fed-batch and continuous cultivation. During the initial 4 weeks, an amount of 50 $mg \cdot l^{-1}$ 4C2MP (final concentration) was fed daily, which was quickly degraded by the biomass. Subsequently, a continuous enrichment procedure was carried out by stepwise increasing the dilution rate from 0.002 to 0.014 h^{-1} and the substrate concentration in the reservoir up to 3.3 $g \cdot l^{-1}$ over a period of 14 weeks.

Measurement of growth

Cell growth was measured turbidometrically at 660 nm using a SPECOL spectrophotometer (Carl Zeiss Jena, Germany). Dry cell mass was estimated from cell suspensions, harvested from the logarithmic phase, washed with distilled water and dried at 100 °C until the weight remained constant. 1 $g \cdot l^{-1}$ dry mass corresponded to $A_{660} = 0.41$.

Base composition of DNA

DNA was isolated and purified from 600 mg freeze-dried cell material as described by Väisänen & Salkinoja-Salonen (1989) with the modification that DNA extraction was carried out repeatedly with chloroform/isoamyl alcohol (24:1 v/v) in the presence of 1 M $NaClO_4$. The guanine-plus-cytosine (G+C) content was analyzed from purified DNA digested to the

nucleoside level according to the method of Tamaoka & Komagata (1984).

Quinone analysis
Quinones were extracted from freeze-dried cells with hexane-methanol (following a protocol of B. J. Tindall, DSM) and separated by HPLC (Millipore Corp.) using a Cosmosil (Nakarai Chemicals LTD, Kyoto, Japan) reversed phase (C18) column (150 mm × 4.6 mm, 5 μm), a mobile phase of methanol : n-butylchloride (80 : 20 v/v), a flow rate of 1 ml·min^{-1} and detection at 269 nm. The ubiquinones were identified comparing their retention times with those of the following reference strains: *Agrobacterium tumefaciens* (DSM 30204) (Q_{10}), *P. fluorescens* (ATCC 11250) (Q_9) and *Serratia marcescens* (Q_8).

Polyamine analysis
Polyamines were extracted and analyzed from exponentially growing cells according to Busse & Auling (1988).

SDS-Polyacrylamide gel electrophoresis of soluble proteins
Cell-free extracts were prepared as described by Straube (1987) from cells grown overnight in nutrient broth, and SDS-PAGE was carried out according to the method of Laemmli (1970), using 16 cm × 17.5 cm slab gels.

Fatty acid analysis
Fatty acids were analyzed according to Väisänen & Salkinoja-Salonen (1989), based on gas chromatography of whole cell fatty acid methyl esters, using a HP 5898 A Microbial Identification System (Hewlett-Packard Company, Avondale, PA, USA) and the MIS System Software 3.2 (Microbial ID, Newark, DE, USA).

Electron microscopy
Cells were negatively stained with 1% aqueous uranyl acetate and observation was carried out with a Tesla BS500 electron microscope.

Phenotypic characterization
Physiological and biochemical properties of strain S1 were investigated by the *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (Braunschweig, Germany) using a standard protocol for routine identification.

Preparation of cell-free extracts
Induced cells were harvested from batch cultures, washed once with 70 mM phosphate buffer (pH 7.9) if 2,4-dichlorophenol hydroxylase and catechol 2,3-dioxygenase was tested or with Tris-hydrochloride buffer (100 mM, pH 8.0) in case of catechol 1,2-dioxygenase. The cell-free extracts were prepared by grinding with Alcoa according to Straube (1987) and enzymes were extracted in the desired buffer used for determinations of enzyme activity. Extracts contained 5 to 10 mg protein per ml. In order to study the substrate specificity of the 2,4-dichlorophenol hydroxylase and to examine the transformation of 5-chloro-3-methylcatechol into 2-methyl-4-carboxymethylenebut-2-en-4-olide, the extract was treated with ammonium sulphate. The fraction between 25 and 55% saturation was resuspended in 67 mM potassium phosphate buffer (pH 7.9) to give a protein concentration of 1.5 to 5 mg·ml^{-1}.

Enzyme assays
2,4-Dichlorophenol hydroxylase can transform also 4C2MP (Beadle & Smith 1982). Its activity was estimated by following the oxidation of NADPH at 339 nm. The standard assay contained (final conc.) 60 mM potassium phosphate (pH 7.9), 4,4 μM FAD, 0.2 mM NADPH, and cell-free extract or the resuspended precipitate of the ammonium sulphate fractionation, respectively. The reaction was started by addition of 0.1 mM phenolic substrate. Catechol 2,3-dioxygenase was assayed by the procedure of Nozaki (1970), catechol 1,2-dioxygenase by the method reported by Dorn & Knackmuss (1978). Activities were determined by photometric tests in a Kontron Uvikon 930 photometer at 30 °C and are expressed as micromoles of substrate converted or product formed per minute per g protein.

Protein estimation
The protein content of cell suspensions and cell-free extracts was determined by the method of Herbert et al. (1971).

Investigation of products from 5-chloro-3-methylcatechol and phenols formed during enzyme reactions in test mixtures
The transformation of 5-chloro-3-methylcatechol (kindly provided by W. Reineke) was investigated in reaction mixtures containing 64 mM potassium phosphate (pH 7.9), 0.08 mM 5-chloro-3-

methylcatechol and partially purified enzyme fractions (0.1 mg protein·ml^{-1}). After consumption of the substrate and accumulation of the 2-methyl-4-carboxymethylenebut-2-en-4-olide (after 4 h), the assay mixture was acidified by 1 M HCl to pH 2 and extracted three times with diethylether. Combined extracts were evaporated, redissolved in bidistilled water, and analyzed by HPLC using methanol : water : acetic acid (30:70:1) as solvent, a programmed flow rate of 0.7 ml·min^{-1} (first 25 min) followed by 1 ml·min^{-1} and detection at 280 nm. Retention times of 5-chloro-3-methylcatechol and 2-methyl-4-carboxymethylenebut-2-en-4-olide were 47.3 and 14.02 min, respectively. Reaction products of the enzymatic conversion of phenols in test mixtures of the 2,4-dichlorophenol hydroxylase assay were extracted in the same manner and analyzed by HPLC using separation conditions as described below. Remaining phenols were quantified by direct injection of the assay mixture into the HPLC system.

Estimation of phenols and intermediates in culture supernatants

Phenol and substituted phenols were analyzed by the 4-aminoantipyrine method (Lacoste et al. 1959). HPLC (LiChrospher RP 8–100 column, 250 mm × 4 mm, 10 μm; mobile phase methanol : water : acetic acid (60:40:1), flow rate 1 ml·min^{-1}) was used for the determination of the substrate specificity of whole cells (detection at 280 nm; Table 2) and to investigate the formation of an intermediate during 4C2MP degradation (detection at 254 nm; Fig. 3). On these conditions the peak of 2-methyl-4-carboxymethylenebut-2-en-4-olide emerged after 3.5 min.

Mass spectrometry

For mass spectrometry 10 ml of cell-free culture fluid was acidified to pH 2 by 1 M HCl and extracted three times with diethyl ether. Combined extracts were dried with Na_2SO_4, evaporated in a stream of N_2 and derivatized with diazomethane (De Boer & Backer 1954). The methyl ester of the intermediate was identified by gas chromatography – mass spectrometry, using a Hewlett-Packard HP 5880 gas chromatograph equipped with a HP-5 crosslinked 5% phenol methylsilicone capillary column (25 m × 0.2 mm × 0.3 μm film thickness) and a HP 5970 A mass selective detector. The peak of the intermediate of the 4C2MP degradation pathway appeared at a retention time of 9.4 min using an oven temperature programme of 0.5 min initial time at 70 °C and increase to 270 °C with a rate of 10 °C per min.

AOX analysis

After removal of cells by centrifugation adsorbable organic halogen was analyzed according to the ISO standard 9562 with an Euroglass AOX analyzer ECS 1000.

DOC and IC analysis

Using a TOC-5000 analyzer (Shimadzu), dissolved organic (DOC) and inorganic carbon (IC) were determined in the culture supernatant after centrifugation (10 min at 6000 rpm). Metabolically produced carbon dioxide in the gas atmosphere was estimated by the following procedure: 500 ml Erlenmeyer flasks containing 200 ml of a cell suspension and 44 mg·ml^{-1} 4C2MP were carefully closed and incubated at 28 °C on a rotary shaker. At certain time intervals the flasks were flushed 5 min with synthetic air (80% nitrogen, 20% oxygen), and the resulting gas stream was bubbled through 5 ml 1 M NaOH to absorb carbon dioxide. Samples were neutralized by 1 M HCl before analysis of IC. All values were corrected by controls lacking 4C2MP.

Results

Enrichment and isolation of a 4-chloro-2-methylphenol degrading bacterium from an industrial waste water treatment plant

The AOX content was analyzed at 4 locations of the Schwarzheide waste water treatment plant to investigate the efficiency of the elimination of chlorinated aromatics at the different waste water purification steps. Samples which were taken from the inlet and the effluent of the aeration tank, and from the effluents of the nitrification and the denitrification step contained 38.8, 0.59, 0.43 and 0.46 mg AOX·l^{-1} (mean values of 2 to 13 samples within a 4 week period), respectively. As indicated by the strong reduction of AOX occurring between inlet and outlet of the aeration tank, most organic-bound halogen had to be mineralized there. This activated sludge which degraded efficiently chloroaromatics was used as inoculum to enrich 4C2MP-degrading bacteria in a chemostat, starting with a fed-batch phase of 4 weeks, followed by continuous cultivation. Using an initial dilution rate

of 0.002 h^{-1} and a 4C2MP concentration of 0.5 $g \cdot l^{-1}$ in the reservoir the biomass was drastically washed out within 20 days (reduction of protein from 2.9 to 0.03 $g \cdot l^{-1}$). However, the concentration of 4C2MP remained below the detection limit in the fermenter fluid. By increasing stepwise its concentration in the reservoir and also the dilution rate, an actively 4C2MP-degrading consortium was enriched, which grew to a density of 0.1 g protein per liter and reached a maximum degradation rate of 0.43 mg 4C2MP $\cdot h^{-1}$ mg $protein^{-1}$.

By plating serial dilutions on nutrient agar, 4 morphological different types of colonies developed and were isolated as pure cultures. All were Gram-negative short rods. The most frequently observed colony type (85% if a dilution rate of 0.0183 h^{-1} was used) showed also in batch experiments the highest degradation ability towards 4C2MP, which was not lost during prolonged subcultivation on nutrient agar I. This strain was called S1 and further characterized in this study.

Phenotypic properties of strain S1

Strain S1 is a Gram-negative, motile, rod-like bacterium, 0.6 to 0.8 μm in width and 1.5 to 3 μm in length. Transmission electron microscopy revealed the existence of a polar flagellum. The strain had the following positive characteristics: growth at room temperature and 40 °C; growth on Mac-Conkey agar; formation of catalase and oxidase; production of acid in ammonium salt medium from glucose, fructose, xylose, and adonitol; degradation of tyrosine; utilization of glycolate, malate, fructose, glucose, mannose, gluconate, N-acetylglucosamine, L-histidine, L-valine, glutarate, and 2-ketoglutarate.

The following features were negative: spore formation, anaerobic growth; growth at pH 5.6 or cetrimide agar; pigment production; oxidation and fermentation of glucose (OF-test); production of acid in ammonium salt medium from rhamnose, mannose, fucose, ribose, arabinose, maltose, cellobiose, lactose, sucrose, melibiose, raffinose, melezitose, inositol, mannitol, sorbitol, salicin, glycerol, and ethanol; hydrolysis of starch, gelatin, casein, DNA, Tween 80, and esculin; production of β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, and lecithinase; nitrate reduction and denitrification; formation of indole and acetoine; utilization of xylose, L-arabinose, maltose, raffinose, mannitol, 2-ketogluconate, acetate, propionate, L-lactate, isobutyrate, n-valerate, caprylate, malonate, adipate, suberate, citrate, levulinate, phenylacetate, D-alanine, glycine, L-leucine, L-serine, citrulline, and tryptamine.

Chemotaxonomy of strain S1

The guanine-plus-cytosine (G+C) content of strain S1 was determined as 61.4 mol%. A ubiquinone with ten isoprenoid units in the side chain (Q-10) was found to be the major quinone system. Spermidine was determined as the major polyamine. The fatty acid profile consisted of 80% C18:1 fatty acids and revealed similarity with *Ochrobactrum anthropi* (similarity index 0.366) and to a less extent with *Xanthobacter agilis*. Therefore, the chemotaxonomic properties of strain S1 were compared with two strains of *O. anthropi* (Table 1). Compared with both strains of *O. anthropi* the patterns of soluble proteins of strain S1 differed somewhat (Fig. 1).

Metabolism of 4-chloro-2-methylphenol

In analogy to the continuously operating waste water treatment plant, growth parameters of strain S1 were determined in chemostat experiments. An increase of biomass yield occurred by enhancing the dilution rate from 0.007 to 0.012 h^{-1} (Fig. 2). Steady state conditions were obtained at dilution rates between 0.012 and 0.02 h^{-1} with a maximum yield coefficient of 0.44 g biomass produced per g substrate. Near the critical dilution rate (approximately 0.021 h^{-1}) 46 μg 4C2MP were consumed per mg biomass and hour.

Cells pregrown on nutrient broth exhibited a lag-phase before the degradation of 4C2MP started in batch cultures. The inducibility of the 4C2MP degradation pathway in strain S1 was evidenced by experiments with chloramphenicol as described in Materials and methods. Using induced cells, breakdown of 0.3 mM 4C2MP started without a lag-phase and was almost completed after 5.5 h (Fig. 3a, b). Total mineralization of 4C2MP was indicated by the decline of the AOX and DOC content and simultaneous formation of carbon dioxide. However, HPLC analysis of the culture fluid revealed the transient excretion of a polar product, which also correlated with the delay of organic carbon decay. After 5.5 h, the value of organic bound halogen (measured as absorbable organic halogen) was zero, indicating that the released compound was already dechlorinated.

Table 1. Chemotaxonomic properties of strain S1 and *O. anthropi* strains LMG 3331^T and 7991.

Features	S1	3331	7991
G+C-content (mol%)	61.4	63.3	62.2
Ubiquinones,			
major component	Q_{10}	Q_{10}	Q_{10}
minor component	Q_9	Q_8	n.d.
Polyamines,			
major component	SPD	SPD	SPD
minor component	DAP, SPM	PUT, DAP	PUT
Fatty acids (%)			
12:0 3OH	0.65	–	–
16:1 *ω7cis*	1.22	0.93	1.34
16:0	4.33	3.09	2.85
17:1 *ω8cis*	2.08	0.25	–
17:1 *ω6cis*	1.01	0.41	0.48
17:0	2.08	1.14	0.74
16:1 3OH	–	0.25	0.47
18:0	2.29	7.92	2.55
19:0 cyclo *ω8cis*	3.92	3.88	1.69
18:1 2OH	–	0.54	2.08
19:0 10-methyl	0.4	–	–
20:3 *ω6,9,12cis*	–	0.31	1.17
20:1 *ω9trans*	1.55	0.5	–
Summed feature 3[a]	–	0.35	0.62
Summed feature 7[b]	80.48	80.43	86.01

[a] Fatty acids 16:1 iso (position of double bound unknown) and 14:0 3OH cannot be separated by using gas chromatography and the MIS system and are designated summed feature 3.
[b] Fatty acids 18:1*trans*6, 18:1*trans*9, and 18:1*cis*11 cannot be separated by using gas chromatography and the MIS system and are designated summed feature 7.
n.d.: not detected, SPD: spermidine, DAP: 1,3-diaminopropane, PUT: putrescine, SPM: spermine.

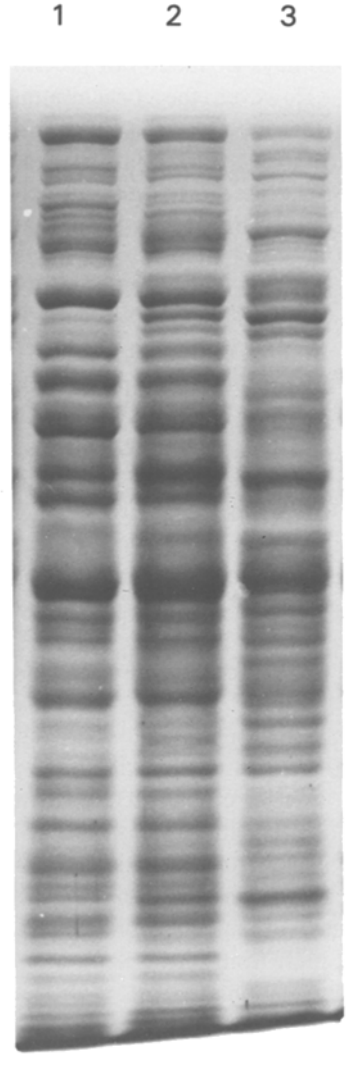

Fig. 1. Pattern of soluble proteins of strain S1 and *O. anthropi* strains in a 10% polyacrylamide gel. The lanes contained 40 μg cell-free extract from *O. anthropi* LMG 7991 (1) and LMG 3331^T (2) and from strain S1 (3).

Isolation and identification of 2-methyl-4-carboxymethylenebut-2-en-4-olide

Formation of this polar intermediate was also observed in cell-free extracts incubated with 5-chloro-3-methylcatechol (a putative intermediate of the 4C2MP degradation pathway) when analyzed by HPLC as described in Materials and methods. Its UV spectrum had a maximum absorbance at 278 nm, which shifted to 250 nm when the pH was adjusted to 13 and was abolished by subsequent acidification to pH 2. The spectral properties were identical to those of 2-methyl-4-carboxymethylenebut-2-en-4-olide, described by Gaunt and Evans (1971) as an intermediate in the metabolism of MCPA by a pseudomonad. The mass spectrum (Fig. 4) of the methylated compound showed the expected molecular ion at *m/z* 168, the base peak at *m/z* 137 ($M^+ - OCH_3$) and a remarkable peak at *m/z* 69 (probably $M^+ - C\text{-}CO\text{-}CH_3$, – CO, involving the rearrangement of a proton), consistent with the mass spectra of the non-derivatized 2-methyldienelactone (Haigler & Spain 1989) and the 2-methyllactone (Pettigrew et al. 1991).

To estimate the released amount of the intermediate, samples were taken from a culture after complete removal of 0.18 mM 4C2MP initially added. At this

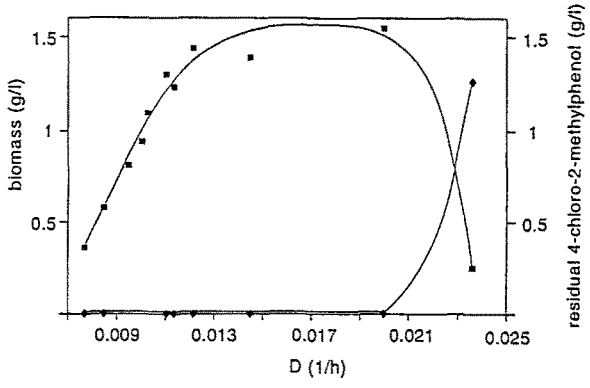

Fig. 2. Growth behaviour of strain S1 in continuous culture. 4C2MP concentration in the influent: 3.5 g·l^{-1}. Biomass formation (■) and 4C2MP concentration (♦) in the fermenter fluid.

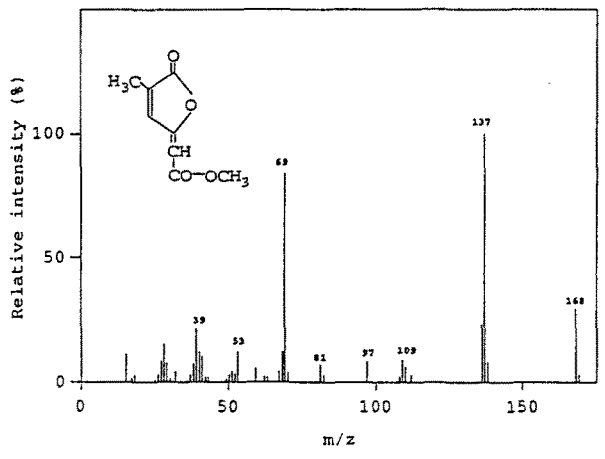

Fig. 4. Mass spectrum of the methylated intermediate.

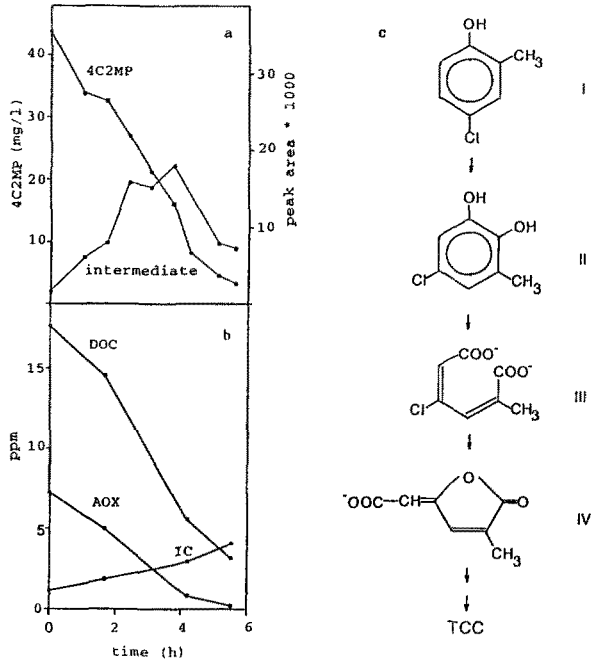

Fig. 3. Time course of mineralization of 4C2MP by strain S1. a: Formation of an intermediate, detected by HPLC (254 nm). b: Mineralization of organically bound carbon and chlorine, determined as described in Materials and methods (representation of the mean values of two to three parallels; IC: cumulative concentration of carbon dioxide in both the gas phase and the medium). Cell density was 0.5 g·l^{-1}. c: Proposed degradation pathway of 4C2MP (I) via 5-chloro-3-methylcatechol (II), 4-chloro-2-methyl-cis,cis-muconate (III) and 2-methyl-4-carboxymethylenebut-2-en-4-olide (IV).

time no other UV-absorbing compound was detectable in the culture supernatant according to HPLC analysis. Using ε_{278} = 19200 M^{-1}·cm^{-1} as given by Gaunt & Evans (1971) 0.018 mM of the 2-methyldienelactone was present at that time.

Degradation of phenols structurally related to 4C2MP

The degradation activity of strain S1 against different phenolic compounds was investigated in batch cultures (Table 2). The herbicides MCPA and 2,4 D did not serve as growth substrates neither for cells pregrown on nutrient broth (data not shown) nor induced by 4C2MP. Cells induced by growth on 4C2MP immediately degraded 2,4-dichlorophenol and 4-chlorophenol with similar rates compared to 4C2MP. In contrast to 4C2MP, no intermediates appeared in the culture medium. Growth on these substrates has not been investigated in batch cultures because initial concentrations of more than 50 mg·l^{-1} inhibited degradation. Lower concentrations did not result in significant biomass increase. Cells converted also 2- and 3-chlorophenol and 2-methylphenol (Table 2). HPLC analyses of culture supernatant revealed, however, that these compounds were not completely removed because degradation stagnated without accumulation of UV-absorbing intermediates. For example, the concentration of 2-methylphenol decreased within 20 h down to 0.04 mM. This amount remained unchanged in the culture fluid over several days. 2,4,6-Trichlorophenol was not attacked.

Cell extracts of strain S1 exhibited for the 2,4-dichlorophenol hydroxylase activity a similar substrate specificity (Table 2). After determinations of the respective rate, the test mixtures were further incu-

Table 2. Substrate spectra of 4-chloro-2-methylphenol-induced cells of strain S1 and of 2,4-dichlorophenol hydroxylase.

Substrate	Degradation rate by 4C2MP induced cells[a] (%)	Specific activity of 2,4-dichlorophenol hydroxylase (%)
2,4-Dichlorophenol	100	100[b]
4-Chloro-2-methylphenol	87	95
4-Chlorophenol	54	56
2-Chlorophenol	16	56
3-Chlorophenol	2	8
2-Methylphenol	5	30
Phenol	10	0
2,4,5-Trichlorophenol	0.3	38
2,4,6-Trichlorophenol	0	0
4-Chloro-2-methylphenoxy-acetic acid (MCPA)	0	n.d.
2,4-Dichlorophenoxyacetic acid (2,4D)	0	0

[a] Setups with 0.5 g dry weight per l were incubated with 0.15 mM of the respective substrate. The degradation rate was calculated from the substrate removal determined by quantitative HPLC-analyses at 280 nm after 1 h (2,4-dichlorophenol, 4C2MP, 4-chlorophenol and phenol) or 3 h (the other compounds tested). 100% corresponded to 0.14 μmoles 2,4-dichlorophenol transformed per h and per mg biomass.
Phenoxyacetic acids were added in final concentrations of 0.4 mM (corresponding to their lower toxicity) and determined by means of their absorbance at 283 nm. No formation of intermediates from 2,4D or MCPA was observed in concomitantly done HPLC analysis at 220 nm.
[b] 100% corresponded to 73 $U \cdot g^{-1}$. n.d.: not determined

bated at 30 °C overnight, and the remaining substrate concentration and the formation of products was estimated by HPLC. 2,4-Dichlorophenol, 4C2MP and 4-chlorophenol were completely metabolized and UV-absorbing intermediates of more hydrophilic nature were detected. 2-Chlorophenol, 3-chlorophenol, 2-methylphenol and 2,4,5-trichlorophenol were still present in the assay mixture with 52, 99, 65 and 52% of the initial concentration, respectively, but also 1 to 2 more hydrophilic products emerged, demonstrating that the investigated phenols were true substrates of the 2,4-dichlorophenol hydroxylase. With phenol, 2,4,6-trichlorophenol and 2,4D no NADPH-oxidizing activity or transformation occurred.

Consumption of phenol by strain S1 started always after a lag phase of 0.5 to 3 h, regardless if the cells were induced by 4C2MP or pregrown on nutrient broth. To establish the induction potential of phenolic compounds towards catabolic pathways, enzyme activities of cells induced by phenol, 2-methylphenol and various chlorinated phenols were determined (Table 3). 4C2MP and 4-chlorophenol induced a relative high level of 2,4-dichlorophenol hydroxylase activity and also a catechol 1,2-dioxygenase activity that converted 4-chlorocatechol at a slightly higher rate compared with catechol. In contrast, incubation with MCPA and 3-chlorophenol resulted in only very low activities of the 2,4-dichlorophenol hydroxylase and zero or almost no activity of the catechol 1,2-dioxygenase(s). On the other hand, phenol and 2-methylphenol induced a considerable activity of a type I catechol 1,2-dioxygenase, which exhibited a higher specificity towards catechol. No appreciable 2,4-dichlorophenol nor phenol hydroxylase activity was present in the latter cells. As indicated by HPLC-analyses of the culture supernatants over 3 days, cells did not metabolize 2-methylphenol through the pathway induced.

In no case a *meta*-cleavage of catechol was found using the catechol 2,3-dioxygenase assay of Nozaki (1970). Additionally, phenol-induced cells were investigated for their type of ring-cleavage using the assay of Ottow and Zolg (1974). 3-Oxoadipate was found as characteristic intermediate of the *ortho*-pathway, whereas the yellow 2-hydroxymuconic semialdehyde, typically of the *meta*-cleavage pathway, did not appear.

Table 3. Induction potential of phenol and chloro- and methyl-substituted phenols towards 2,4-dichlorophenol hydroxylase and catechol 1,2-dioxygenase activities of cells from strain S1.

Enzyme activity	Assay substrate	Specific activities[a] (U·g protein^{-1}) after induction with					
		4C2MP	4CP	3CP	MCPA	Phenol	2MP
2,4-Dichlorophenol hydroxylase	2,4-Dichlorophenol	26	63	1	4	1	0
	Phenol	< 1	< 1	< 1	0	0	0
Catechol 1,2-dioxygenase	4-Chlorocatechol	16	111	< 3	< 1	99	41
	Catechol	14	88	3	0	896	156

[a] Activities were determined by photometric tests using cell-free extracts. For induction of the catabolic enzymes, cells harvested from nutrient broth were resuspended in mineral salt solution in the presence of 0.1 g·l^{-1} yeast extract and 0.15 mM of the respective substrate. After an incubation period of 2 to 3 h cells were used for the preparation of cell-free extracts (see also the Materials and methods section). 4CP/3CP: 4-, 3-chlorophenol; 2MP: 2-methylphenol.

Discussion

Taxonomical investigations

Some Gram-negative bacteria, belonging to the *Proteobacteria*, have been described in the literature that utilize chlorinated phenoxyalkanoic acids and their chlorophenol derivatives , i.e., *Alcaligenes eutrophus* JMP 134 (Pieper et al. 1988; β-subclass) or less characterized strains of the genera *Acinetobacter* (Beadle and Smith 1982; γ-subclass), *Pseudomonas* (Karns et al. 1983, Gaunt and Evans 1971; γ-subclass), and *Xanthobacter* (Ditzelmüller et al. 1989; α-subclass).

The chemotaxonomic data presented here clearly allow an allocation of the 4C2MP-degrading strain S1 to the α-2 subclass (Busse & Auling 1988, Auling 1993). *Ochrobactrum anthropi* is also a member of this subgroup and was indicated by the MID 3.30 software to be most similar to strain S1. The values of G+C content of strain S1 and of the two strains of *O. anthropi* were in the same range. Perhaps due to the determination by HPLC, the values were slightly higher than originally reported using thermal melting (Holmes et al. 1988). However, at present we would not be inclined to group strain S1 into *O. anthropi*, because this organism differed in a number of physiological reactions (absence of nitrate reduction, no utilization of e.g. acetate, isobutyrate, n-valerate, mannitol, 2-ketogluconate, L-serine, L-leucine, D-alanine and glycine) from the latter (Holmes et al. 1988).

Biodegradative pathway of 4C2MP

Only some information is available on degradation of 4C2MP as sole carbon source, whereas microbial degradation of the herbicide MCPA was investigated by different authors (Bollag et al. 1967, Gaunt & Evans 1971, Pieper et al. 1988) and was described to proceed via 4C2MP. Pieper et al. (1989) studied the induction capacity of 4C2MP and 2,4-dichlorophenol regarding to the regulation of the catabolic pathways of chloroaromatics and phenol in *A. eutrophus* JMP 134. Therefore, the degradative properties of strain S1 have to be discussed compared with strain JMP 134. MCPA induced cells of strain JMP 134 metabolized 4C2MP, whereas 4C2MP itself did not show an induction potential and was consequently not attacked when added as sole carbon source. In contrast, strain S1 failed to utilize MCPA at all, although it had been isolated from activated sludge that eliminated phenoxyalkanoic acids. In strain S1 4C2MP and 4-chlorophenol (or respective intermediates of the degradation pathway) were found to induce a 2,4-dichlorophenol hydroxylase and a catechol 1,2-dioxygenase similar to type II of the "chloroaromatic pathway" according to the ratio of 4-chlorocatechol and catechol conversion (Pieper et al. 1988). Further evidence for the operation of a modified *ortho*-cleavage pathway in strain S1 (Fig. 3c) was obtained by identification of 2-methyl-4-carboxymethylenebut-2-en-4-olide as intermediate of the conversion of 4C2MP by whole cells and of 5-chloro-3-methylcatechol by cell-free extract of strain S1. Release of 2-methyl-4-carboxymethylenebut-2-en-4-olide into the culture fluid has been described for biodegradation of MCPA (Gaunt & Evans 1971) and for the cometabolic breakdown of p-chlorotoluene (Haigler & Spain 1989) by different pseudomonads.

Phenol and 2-methylphenol were converted only unsufficiently by this metabolic route (Table 2). Sim-

ilar to JMP 134, strain S1 appeared to utilize phenol through an alternative, independently induced pathway. While in strain JMP 134 both *meta*- and *ortho*-cleavage activities were expressed during growth on phenol (Pieper et al. 1989), strain S1 exhibited only the *ortho*-pathway. This fact may be the result of a selection process and an effective adaptation to the conditions in the waste water dominated by chlorinated aromatics. The lack of *meta*-pathway enzymes seems to have the advantage to prevent a "misrouting" of chloroaromatics into suicide metabolites or dead-end products (Bartels et al. 1984). Strain S1 was isolated as a member of the actively phenoxyalkanoic acids mineralizing activated sludge. Organisms with an inducible degradation pathway for chlorophenols may play an important role in protecting the microbial community against accumulation of toxic intermediates during the breakdown of the herbicidal waste water components.

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