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Degradation of 4-Chlorophenol via the *meta* Cleavage Pathway by *Comamonas testosteroni* JH5

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***Comamonas testosteroni* JH5 used 4-chlorophenol (4-CP) as its sole source of energy and carbon up to a concentration of 1.8 mM, accompanied by the stoichiometric release of chloride. The degradation of 4-CP mixed with the isomeric 2-CP by resting cells led to the accumulation of 3-chlorocatechol (3-CC), which inactivated the catechol 2,3-dioxygenase. As a result, further 4-CP breakdown was inhibited and 4-CC accumulated as a metabolite. In the crude extract of 4-CP-grown cells, catechol 1,2-dioxygenase and muconate cycloisomerase activities were not detected, whereas the activities of catechol 2,3-dioxygenase, 2-hydroxymuconic semialdehyde dehydrogenase, 2-hydroxymuconic semialdehyde hydrolase, and 2-oxopent-4-enoate hydratase were detected. These enzymes of the *meta* cleavage pathway showed activity with 4-CC and with 5-chloro-2-hydroxymuconic semialdehyde. The activities of the dioxygenase and semialdehyde dehydrogenase were constitutive. Two key metabolites of the *meta* cleavage pathway, the *meta* cleavage product (5-chloro-2-hydroxymuconic semialdehyde) and 5-chloro-2-hydroxymuconic acid, were detected. Thus, our previous postulation that *C. testosteroni* JH5 uses the *meta* cleavage pathway for the complete mineralization of 4-CP was confirmed.**

The aerobic microbial degradation of many aromatic compounds occurs via catechols as key metabolites. The breakdown of catechols proceeds via the central step of *ortho* or *meta* ring fission. In general, methyl-substituted aromatic compounds are degraded via the *meta* cleavage pathway, whereas xenobiotics like chloroaromatic compounds are mineralized via the *ortho* cleavage pathway (9, 21). The degradation of methyl- and chloroaromatic substrates in mixture is often incomplete, leading to the accumulation of dead-end metabolites like chlorocatechols, chlorinated hydroxymuconic semialdehydes, or 4-carboxymethyl-methylbut-2-en-1,4-olides (31, 33). As a possible metabolite of chloroaromatic breakdown, 3-chlorocatechol (3-CC) reversibly inhibits the *meta*-cleaving catechol 2,3-dioxygenase or totally blocks the *meta* cleavage pathway by the irreversible suicide inactivation of this dioxygenase (4, 16, 20).

Recently, we described *Comamonas testosteroni* JH5, which completely mineralizes a mixture consisting of 4-chlorophenol (4-CP) and monomethylphenols (16). The degradation of 4-methylphenol (4-MP) and 4-CP occurred successively. Mixtures consisting of 4-CP and 2-MP or 3-MP were mineralized simultaneously. Since a catechol 2,3-dioxygenase, but no *ortho*-cleaving enzyme, was detected in cells grown on 4-CP, the mineralization of 4-CP via *meta* ring fission was postulated (16). Furthermore, a *Pseudomonas* species is able to completely mineralize 5-chlorovanillate via *meta* cleavage (18). Kersten et al. (18) showed that the formed acylchloride undergoes cyclization, which leads to a spontaneous removal of the chloride substituent. This mechanism is restricted to *ortho*-substituted halogens and therefore would not work with 4-CP. Recently, the *meta* cleavage of 4-chlorobenzoate by *Pseudomonas cepacia* P166 was shown, and the key metabolites of the *meta* cleavage pathway of 4-chlorobenzoate were identified (1, 2). The following experiments were designed to prove the use of the *meta* cleavage pathway for 4-CP by strain JH5.

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MATERIALS AND METHODS

Chemicals. All chemicals were of the highest purity available. 4-CC was obtained from Helix Biotech Corporation (Canada), 3-CC was obtained from Aldrich Chemie GmbH & Co. KG (Steinheim, FRG), *cis,cis*-muconic acid was a gift from K.-H. Engesser (University of Stuttgart, Stuttgart, Federal Republic of Germany), and 2-chloro-*cis,cis* muconic acid was a gift from M. Schlömann (University of Stuttgart).

2-Hydroxymuconic semialdehydes were prepared enzymatically, supplying the corresponding catechols (0.08 mM) in 50 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.5) and a crude cell extract of strain JH5 with an active catechol 2,3-dioxygenase. The activity of the enzymes, which catalyze the transformation of the semialdehydes, was destroyed by a heat treatment of the crude cell extract (56°C for 8 min).

2-Oxopent-4-enoic acid was prepared enzymatically by converting DL-allylglycine with an L-amino acid oxidase. DL-Allylglycine (120 mM) was incubated overnight in a reaction mixture consisting of 33 mM KCl, 23 mM Tris-(hydroxymethyl)-aminomethane-hydrochloride buffer (pH 7.2), and 0.016 mg of L-amino acid oxidase per ml. The reaction was stopped by the addition of perchloric acid to a final concentration of 0.25% (vol/vol). After centrifugation, the clear supernatant was used immediately in the 2-oxopent-4-enoate hydratase assays.

3-Chloro-*cis,cis*-muconic acid was prepared from 4-CC with the catechol 1,2-dioxygenase in cell extract of *Alcaligenes xylosoxidans* subspecies *denitrificans* JH1. The catechol 2,3-dioxygenase of strain JH1 was destroyed by a pretreatment of the crude extract with H_2O_2 (0.01%) for 5 min. 4-CC (0.12 mM) in Tris-buffer (50 mM [pH 7.5]; 1 mM $MnCl_2$) was transformed completely to the corresponding 3-chloro-*cis,cis*-muconic acid by the repeated addition of a crude extract from strain JH1. The production of 3-chloro-*cis,cis*-muconic acid was monitored by measuring the extinction at 260 nm. The reaction was stopped by heating the mixture for 20 min at 56°C. After centrifugation, the clear supernatant was used immediately in the cycloisomerase tests.

Bacterial strain and culture conditions. Enrichment, identification, and growth of *C. testosteroni* JH5 were described previously (16). Strain JH5 was routinely grown at 27°C in a minimal medium with 4-CP (1 mM) and 4-MP (1 mM) in order to maintain specific degradation ability.

In batch culture studies, 4-CP or mixtures of isomeric chlorophenols were the sole sources of energy and carbon, and strain JH5 grown on 4-CP was used for inoculation (10%). The assay mixtures were incubated in the dark at 25°C on a rotary shaker.

Assays with resting cells were performed with cells grown on 4-CP and harvested during the late phase of exponential growth. Then the cells were centrifuged, washed, and resuspended in 50 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.5). After the addition of substrate, the cells were incubated at 22°C. Enzyme assays were performed with strain JH5 grown on phenolic substrates (1 mM) or acetate (5 mM) to the late exponential phase.

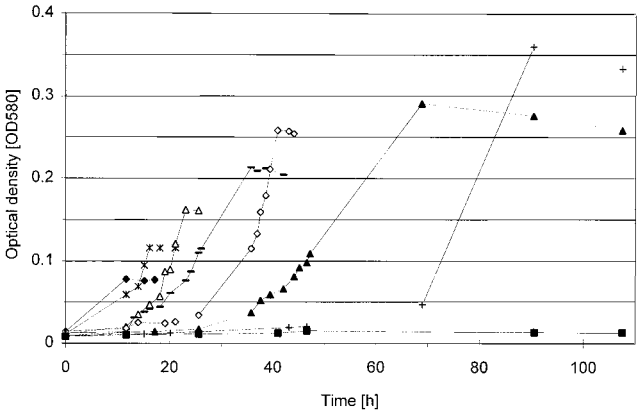

FIG. 1. Growth curves of *C. testosteroni* JH5 at different concentrations of 4-CP, the sole source of carbon and energy. Symbols: ◆, 0.25 mM; ×, 0.5 mM; △, 0.75 mM; ——, 1 mM; ◇, 1.25 mM; ▲, 1.5 mM; +, 1.75 mM; ■, 2 mM 4-CP.

Analytical measurements. Phenolic substrates and metabolites were analyzed by high-performance liquid chromatography (HPLC) as described elsewhere (16). Chloride release was measured by potentiometric detection with an ion-selective Ag/AgCl electrode (12). Cell growth was monitored by measurement of the optical density at 580 nm in a spectrophotometer (Philips 8625). The protein concentration of whole cells was determined as described previously (16).

Isolation and identification of metabolites of the *meta* cleavage pathway. The metabolites were prepared enzymatically with a crude extract of cells grown on 1 mM 4-CP. For the isolation of 5-chloro-2-hydroxymuconic semialdehyde, 5 μmol of 4-CC was incubated in a total volume of 50 ml of K_2HPO_4-KH_2PO_4 buffer (10 mM, pH 7.5) with the crude extract (12.5 mg of protein). The reaction was immediately heat inactivated (at 56°C for 30 min) when the extinction at 375 nm remained constant. 5-Chloro-2-hydroxymuconic acid was prepared in the same manner, but NAD^+ was added to the reaction mixture at a final concentration of 0.2 mM. As the yellow color of the solution disappeared, the reaction was heat inactivated. After the solutions containing the metabolites were cooled, their pHs were adjusted to 3 by the addition of hydrochloric acid. The solutions were centrifuged, and the supernatants were extracted three times with diethyl ether. Combined extracts were gassed with nitrogen to dryness, and the residues were resolved in methanol. The samples were analyzed by HPLC (model HP 1100 chromatograph; Hewlett-Packard) with UV diode-array detection (DAD) and electrospray ionization mass spectrometry (ESI-MS) detection (model SSQ 7000 spectrometer; Finnigan MAT). A reversed-phase C_{18} column was used (particle size, 3 μm; 2 × 125 mm; Merck) with methanol and H_2O–1% acetic acid as the mobile phase at a flow rate of 0.3 ml/min. A gradient from 5 to 50% methanol within 10 min was employed. A voltage of −4.4 V was applied to the electrospray needle.

Enzyme assays. Cells of strain JH5 were harvested and broken by ultrasonic treatment as previously described (16). The protein content of the cell extracts was determined according to the method of Bradford (6). Enzymatic activities of catechol 1,2-dioxygenase (EC 1.13.11.1) and catechol 2,3-dioxygenase (EC 1.13.11.2) were determined according to the methods of Nakazawa (26) and Nozaki (27), respectively. Simultaneously present catechol 2,3-dioxygenase, which interferes with the determination of catechol 1,2-dioxygenase activity, was destroyed by incubating the crude extract for 5 min with H_2O_2 (0.01%). Subsequent cycloisomerization of the *cis,cis*-muconic acid formed by the activity of the catechol 1,2-dioxygenase was prevented by the addition of EDTA to a final concentration in the assay of 1.3 mM. The extinction coefficient of 2-hydroxy-5-methylmuconic semialdehyde was that of Bayly et al. (5). The conversion of 4-CC was measured with spectral data determined previously (16).

The 2-hydroxymuconic semialdehyde hydrolase and 2-hydroxymuconic semialdehyde dehydrogenase were assayed according to the method of Hopper and Taylor (17). The enzyme assays contained 0.04 mM freshly produced 2-hydroxymuconic semialdehydes (see chemicals listed above) in a solution containing 25 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.5) and crude cell extract. The dehydrogenase assays also contained NAD^+ at a concentration of 0.2 mM. The dehydrogenase activity was calculated by subtracting the measured hydrolase activity from that of extracts assayed in the presence of NAD^+.

The 2-oxopent-4-enoate hydratase was assayed according to the method of Collinsworth et al. (8). The enzyme assays contained 60% (vol/vol) of the freshly produced 2-oxopent-4-enoic acid in a 33 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.5) with 0.33 mM $MnCl_2$ and crude cell extract to provide an initial absorption of 2 absorption units at 265 nm. The extinction coefficient of 2-oxohex-4-enoic acid (ε = 10,000 M^{-1} cm^{-1}) was used for the calculations, assuming that 2-oxohex-4-enoic acid absorbs light in the same manner as 2-oxopent-4-enoic acid (8).

The muconate cycloisomerase activity was determined according to the method of Ornston (28) with *cis,cis*-muconic acid, 2-chloro-*cis,cis*-muconic acid, and freshly produced 3-chloro-*cis,cis*-muconic acid (see chemicals listed above) as substrates. The extinction coefficients of the muconic acids were those of Dorn and Knackmuss (10).

The activity of phenol hydroxylase was determined by measuring polarographically the oxygen uptake rates of resting cells (0.18 mg of protein per ml) with phenolic substrates (0.1 mM) at 25°C by using a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). The rates obtained were corrected for endogenous consumption. The O_2 uptake rate represents the total activity of phenol hydroxylases and catechol dioxygenases. Since the rates with catechols were significantly faster than the rates with phenols (data not shown), the activity of phenol hydroxylases was considered to be rate limiting, and half of the O_2 uptake rate was assumed to correspond to the phenol hydroxylase activity.

All enzyme tests were performed in triplicate, with at least two crude extracts from different cultures. The maximum deviation of the activity values was 15%.

RESULTS

Chlorophenol degradation by *C. testosteroni* JH5. The growth of strain JH5 with 4-CP as its sole source of energy and carbon was tested, with the concentration of 4-CP ranging from 0 to 2.38 mM. Increasing 4-CP concentration promoted growth, as shown in Fig. 1. Complete mineralization of 4-CP was observed up to a substrate concentration of 1.8 mM. Stoichiometric amounts of chloride were released (Fig. 2), and no UV-detectable metabolites accumulated. Control assays without 4-CP showed no growth or release of chloride, and sterile controls showed no abiotic 4-CP loss. An increase in the 4-CP concentration caused a toxic effect, which became obvious with increased lag times (Fig. 1). A concentration of 2.0 mM inhibited growth completely; within 14 days of incubation, no growth and only low chloride release were observed (Fig. 1 and 2).

2-CP and 3-CP were not the sole sources of energy and carbon. Mixed with 4-CP as the growth substrate, both chlorophenols were partly transformed. If 0.25 mM 2-CP or 3-CP was mixed with an equimolar amount of 4-CP, 4-CP was transformed completely, whereas the degradation of the isomeric chlorophenols was incomplete (70%) and only 0.25 mM chloride was released. The amount of chloride that was released decreased even more as the concentrations of the chlorophenols in the mixture were increased to 0.5 mM, an effect obviously caused by an inhibitory effect of 2-CP and 3-CP. Because of these results, 2-CP was used in additional experiments as an inhibitory substrate for 4-CP degradation to detect metabolites from 4-CP. Assays were conducted with resting cells grown on

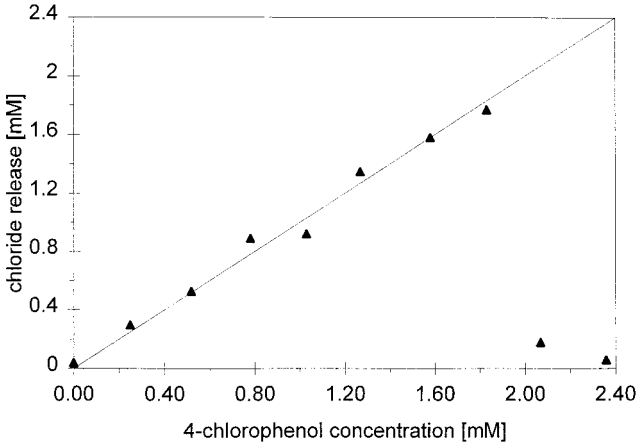

FIG. 2. Chloride release by *C. testosteroni* JH5 at different concentrations of 4-CP, the sole source of carbon and energy (corresponding growth curves are shown in Fig. 1). Symbols: ▲, chloride release; ——, stoichiometric release of chloride.

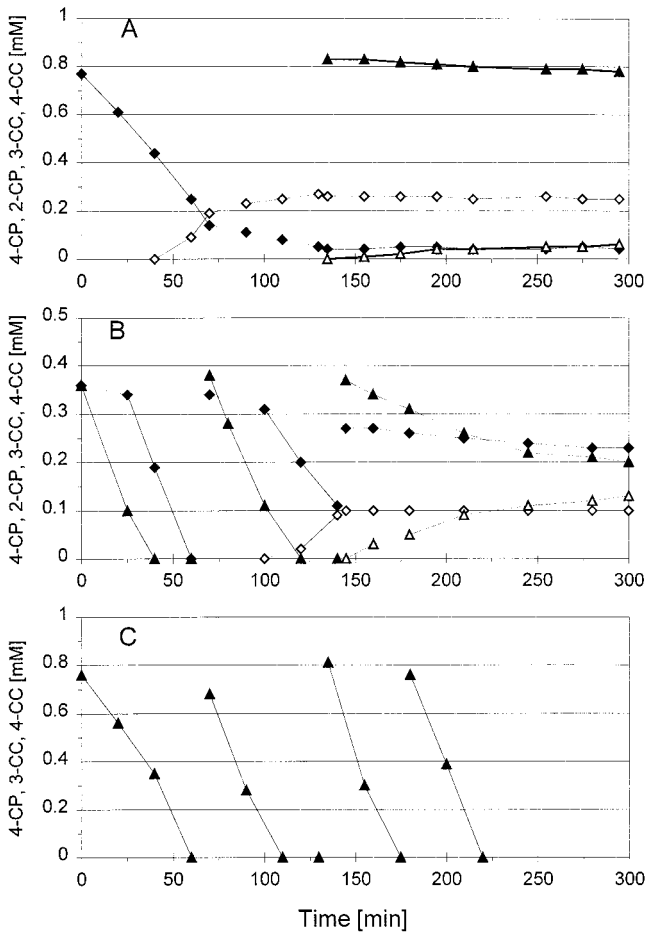

FIG. 3. Degradation of 2-CP and 4-CP by resting cells of *C. testosteroni* JH5 grown on 4-CP (0.41 mg of protein per ml). Symbols: ▲, 4-CP; ◆, 2-CP; △, 4-CC; ◇, 3-CC. (A) 2-CP supplied at the beginning and 4-CP added later; (B) 2-CP supplied in mixture with 4-CP three times during the assay; (C) 4-CP supplied four times during the assay (control).

4-CP (0.41 mg of protein per ml) (Fig. 3). The addition of 0.77 mM 2-CP to the resting cells resulted in an almost complete (95%) transformation of this compound (Fig. 3A). Formation of 3-CC started after half of the 2-CP had been transformed. With a progressive turnover of 2-CP, 3-CC accumulation increased up to a maximum concentration of 0.25 mM. The formation of 3-CC was not stoichiometric; this was obviously due to the *meta* cleavage of 3-CC, which resulted in the inactivation of the catechol 2,3-dioxygenase. As a consequence, after 130 min, the added 4-CP (0.83 mM) was transformed only to a minor extent. 4-CC was formed in equimolar amounts as 4-CP was consumed (0.06 mM). Figure 3B shows another assay supplying an equimolar mixture of 2-CP and 4-CP (0.35 mM each) to the resting cells. First, both compounds were degraded without the formation of chlorocatechols. 4-CP degraded faster than 2-CP. The addition of more of the 2-CP–4-CP mixture to the cells resulted in a complete turnover of 4-CP. 2-CP transformation was slower, and 3-CC accumulated. As the mixture was added a third time, 2-CP was transformed only to a very small extent, and the rate of 4-CP degradation slowed, stopping after 300 h. 4-CC formation to a concentration of 0.12 mM was observed. The medium for both assays, shown in Fig. 3A and B, turned pink, indicating that reactions of the formed chlorocatechols, such as oxidation and polymerization, had occurred, as shown by Fava et al. (11) and Haller and Finn (13). In contrast, control experiments with resting cells (Fig. 3C) showed that successive additions of 4-CP (0.75 mM) resulted in the complete mineralization of 4-CP, accompanied by the stoichiometric release of chloride (data not shown).

Identification of the metabolites of the *meta* cleavage pathway. The metabolites were prepared enzymatically from 4-CC by a crude extract of cells grown on 4-CP. After extraction from the assay fluid, identification was done by HPLC–ESI-MS and HPLC–UV-DAD. The negative-ion mass spectrum of 5-chloro-2-hydroxymuconic semialdehyde (Fig. 4A) received at a retention time of 7.0 min was the largest peak of the UV chromatogram (350 nm) detected. The peak at *m/z* 175 corresponds to the pseudomolecular ion $[M - H]^-$ as is typical for ESI with negative-ion detection. The signal of the fragment ion at *m/z* 103 $[M - CO\text{-}COOH]^-$ was increased as the collision-induced dissociation (CID) was raised to 20 V. Both ion signals are accompanied by a smaller $n + 2$ peak in a ratio consistent with the presence of a single chlorine atom. The UV spectrum of the semialdehyde showed an absorption maximum at 378 nm at a neutral pH as reported elsewhere (30, 34).

The mass spectrum of 5-chloro-2-hydroxymuconic acid received at a retention time of 6.3 min was the largest peak of the UV chromatogram (275 nm) detected. The mass spectrum gave $[M - H]^-$ at *m/z* 191, $[M - COOH]^-$ at *m/z* 147, and $[M - CO\text{-}COOH]^-$ at *m/z* 119 (Fig. 4B). Increases in the signals of the fragment ions *m/z* 147 and *m/z* 119 occurred with increasing CID voltage. The UV spectrum at the peak at 6.8 min showed good correspondence with the spectrum of

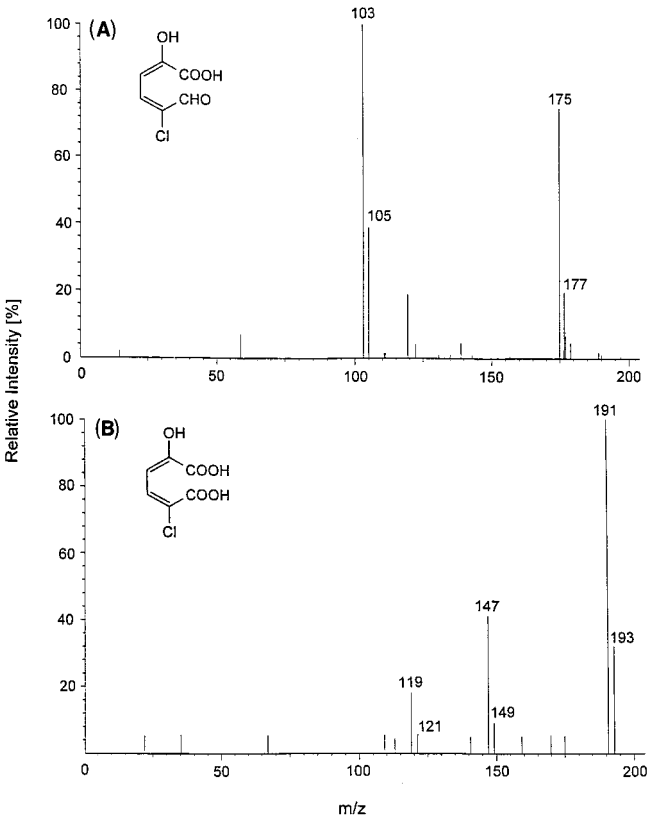

FIG. 4. Negative-ion mass spectra of metabolites of the *meta* cleavage pathway of 4-CP obtained by HPLC–ESI-MS. (A) 5-chloro-2-hydroxymuconic semialdehyde (CID, 20 V); (B) 5-chloro-2-hydroxymuconic acid (CID, 10 V).

TABLE 1. Specific activities of phenol hydroxylase in cells of *C. testosteroni* JH5

Assay substrate	Sp act (mU/mg of protein) after growth on:		
	Acetate	Phenol	4-CP
2-CP	<1	24	52
3-CP	<1	12	35
4-CP	<1	67	157
4-Bromophenol	<1	50	84
4-Chloro-3-MP	<1	69	92
2,4-Dichlorophenol	<1	13	11

2-chloromuconic acid, with absorption maxima at 280 nm (chloro-hydroxymuconic acid) and 275 nm (chloromuconic acid) at low pH. The mass spectra of both metabolites cannot explain whether the *meta* cleavage of 4-CC was proximal or distal. However, the measured enzyme activities and observations elsewhere (2, 34) indicate proximal *meta* cleavage.

Enzyme activities. A phenol hydroxylase was induced in cells of strain JH5 grown on phenol or 4-CP (Table 1). The hydroxylase catalyzed the turnover of all three isomeric chlorophenols and the other phenols tested. The highest specific activity was measured in crude extract of 4-CP-grown cells, with 4-CP as the assay substrate.

Whereas the formation of phenol hydroxylases was induced (Table 1), the *meta*-cleaving catechol 2,3-dioxygenase was formed constitutively because of high activities also measured in cells grown on acetate (Table 2) and succinate (data not shown). The enzyme exhibited the highest activity when catechol was used as the substrate, followed by 4-methylcatechol. Less catechol 2,3-dioxygenase activity was detected when 4-CC and 3-MC were used as substrates. No activity at all was measured with 3-CC due to the irreversible inactivation of the catechol 2,3-dioxygenase.

Regardless of the growth substrate and growth phase in which the cells were harvested, no catechol 1,2-dioxygenase activity was detected (Table 2). A chloromuconate cycloisomerase, catalyzing the further turnover of *ortho* ring fission products of catechols, was not detected either. The suitability of the assays was proved with the crude extract of a *Pseudomonas* strain which possesses a catechol 1,2-dioxygenase and a chloromuconate cycloisomerase. Instead of these enzymes, a 2-hydroxymuconic semialdehyde hydrolase and dehydrogenase, catalyzing the further turnover of *meta* ring fission products of catechols, were present (Table 3). The dehydrogenase

TABLE 2. Specific activities of catechol dioxygenases in the crude cell extract of *C. testosteroni* JH5

Enzyme assayed and assay substrate	Sp act (mU/mg of protein) after growth on:				
	Acetate	Phenol	2-MP	4-MP	4-CP
Catechol 2,3-dioxygenase					
Catechol	1,726	1,613	1,551	1,452	1,781
3-Methylcatechol	203	109	150	200	137
4-Methylcatechol	503	464	461	494[a]	419[a]
3-CC	<1	<1	<1	<1	<1
4-CC	174	129	118	135[a]	173[a]
Catechol 1,2-dioxygenase					
Catechol	<1	<1	<1	<1	<1
4-CC	<1	<1	<1	<1	<1

[a] Value has been published previously (14).

TABLE 3. Specific activities of 2-hydroxymuconic semialdehyde hydrolase and dehydrogenase in the crude cell extract of *C. testosteroni* JH5

Enzyme assayed and assay substrate	Sp act (mU/mg of protein) after growth on:		
	Acetate	Phenol	4-CP
2-Hydroxymuconic semialdehyde hydrolase			
2-Hydroxymuconic semialdehyde	<0.5	36	35
2-Hydroxy-5-methylmuconic semialdehyde	<0.5	16	18
5-Chloro-2-hydroxymuconic semialdehyde	<0.5	4	7
2-Hydroxymuconic semialdehyde dehydrogenase			
2-Hydroxymuconic semialdehyde	282	493	330
2-Hydroxy-5-methylmuconic semialdehyde	76	245	261
5-Chloro-2-hydroxymuconic semialdehyde	32	84	63

was expressed constitutively, whereas the hydrolase was induced only in phenol- or 4-CP-grown cells (Table 3). The NAD^+-dependent enzyme exhibited an activity at least 10-fold higher than that of the hydrolase. Both enzymes transformed 2-hydroxymuconic semialdehyde faster than the substituted analogs 5-chloro-2-hydroxymuconic semialdehyde and 2-hydroxy-5-methylmuconic semialdehyde. 2-Oxopent-4-enoate hydratase, an enzyme in the later part of the *meta* pathway, was detected in the crude extract of 4-CP-grown cells. The hydratase exhibited an activity of 415 mU of protein per ml, with 2-oxopent-4-enoate used as the assay substrate.

DISCUSSION

The present studies show that compared with other strains described elsewhere, *C. testosteroni* JH5 is able to tolerate and degrade rather high concentrations of 4-CP used as the sole source of energy and carbon (3, 32). *Pseudomonas* sp. strain B13 failed to grow if 4-CP was supplied at a concentration above 1 mM (32), and *Alcaligenes* sp. strain A7-2 did not tolerate concentrations above 1.24 mM (3).

Figure 5 shows the proposed metabolic pathway for the degradation of 4-CP by strain JH5, consistent with the results of this study. Degradation starts with the attack of a phenol hydroxylase (Fig. 5, enzyme A), which exhibits a broad substrate specificity. The enzyme catalyzes the turnover of 2-CP and 3-CP even though both chlorophenols are not the sole growth substrates for this strain. 4-CC, the oxidation product of 4-CP, was not turned over by *ortho* cleavage, because neither catechol 1,2-dioxygenase nor muconate cycloisomerase activity could be detected. In contrast, cells of JH5 contained a constitutively formed catechol 2,3-dioxygenase (enzyme B), which exhibited high activity with 4-CC (Table 2). The dioxygenase activities obtained with one assay substrate are fairly constant, regardless of the growth substrate used (Table 2). This indicates that only one *meta*-cleaving dioxygenase is present in strain JH5. Since the catechol 2,3-dioxygenase was irreversibly inactivated by 3-CC, 2-CP and 3-CP were not used as growth substrates. The broad substrate specificity of the phenol hydroxylase induced on 4-CP, however, accounts for the cometabolic formation of 3-CC from 2-CP or 3-CP. This was shown by tests with resting cells (Fig. 3A and B) as well as by additional enzyme assays (Table 1). 3-CC and 4-CC accumulated in the presence of 2-CP and 4-CP as a substrate mixture, and further 4-CP breakdown was irreversibly inhibited. Therefore, the possibility of another metabolic turnover of 4-CP, such as a hydrolytic or an oxygenolytic elimination of chloride, as shown

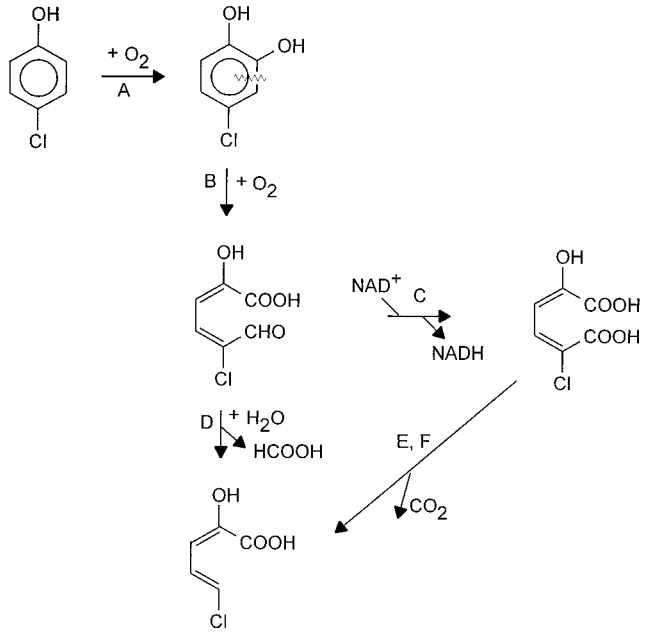

FIG. 5. Proposed pathway for the metabolism of 4-CP by *C. testosteroni* JH5. A, phenol hydroxylase; B, catechol 2,3-dioxygenase; C, 2-hydroxymuconic semialdehyde dehydrogenase; D, 2-hydroxymuconic semialdehyde hydrolase; E, 4-oxalocrotonate isomerase; F, 4-oxalocrotonate decarboxylase.

for other chlorinated aromatic compounds (19, 23–25), can be excluded.

Other investigations have indicated that a *meta*-cleaving catechol 2,3-dioxygenase transforms 4-CC to 5-chloro-2-hydroxymuconic semialdehyde, which accumulates in the culture liquid and becomes visible by its yellow color (7, 15, 29, 31–34). In contrast, 4-CP breakdown via *meta* cleavage in strain JH5 resulted in complete mineralization; no semialdehydes were accumulated, and chloride was released in stoichiometric amounts. Sometimes the culture medium turned yellow for a short period of time, indicating the transient formation of 5-chloro-2-hydroxymuconic semialdehyde. The yellow color, with an absorption maximum at 378 nm, disappeared upon acidification, as described by Wieser et al. (34) for 5-chloro-2-hydroxymuconic semialdehyde. Strain JH5, however, is able to degrade this compound further. The identification of the enzymatically produced 5-chloro-2-hydroxymuconic semialdehyde was achieved by HPLC–DAD-MS (Fig. 4).

The turnover of 5-chloro-2-hydroxymuconic semialdehyde in strain JH5 is catalyzed by a 2-hydroxymuconic semialdehyde dehydrogenase and a 2-hydroxymuconic semialdehyde hydrolase (Fig. 5, enzymes C and D). The NAD^+-dependent dehydrogenation seems to be more significant than the hydrolysis, because the dehydrogenase exhibits a much higher activity than the hydrolase (Table 3). This observation is in accordance with data for the nonchlorinated substrate (14, 30). The induction of the hydrolase with phenols as growth substrates, however, indicates that the hydrolase step is also of physiological significance. In *P. cepacia* P166, the formation of metabolites from 4-chlorobenzoate indicated that the dehydrogenative branch of the *meta* pathway is more significant than the hydrolytic branch, as is the case in strain JH5. 5-Chloro-4-hydroxy-2-oxopentanoic acid produced in strain P166 is cleaved to pyruvate and chloroacetate (1, 2). Chloride release by this strain is slow and does not coincide with 4-chlorobenzoate turnover, because chloroacetate accumulated intermediately. In contrast, the breakdown of 4-CP by strain JH5 is fast, and chloride release coincides as previously reported (16). No metabolites accumulated for a longer period of time in growth cultures or resting cells. Thus, it would be interesting to investigate which step in the transformation is responsible for chloride elimination.

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