Biodegradation **11:** 247–261, 2000.

Pathways for 3-chloro- and 4-chlorobenzoate degradation in *Pseudomonas aeruginosa* 3mT

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Accepted 2 August 2000

Key words: 3-chlorobenzoate, 4-chlorobenzoate, chlorocatechols, modified *ortho*-pathway, *Pseudomonas aeruginosa*

Abstract

A bacterial isolate, *Pseudomonas aeruginosa* 3mT exhibited the ability to degrade high concentrations of 3-chlorobenzoate (3-CBA, 8 g l^{-1}) and 4-chlorobenzoate (4-CBA 12 g l^{-1}) (Ajithkumar 1998). In this study, by delineating the initial biochemical steps involved in the degradation of these compounds, we investigated how this strain can do so well. Resting cells, permeabilised cells as well as cell-free extracts failed to dechlorinate both 3-CBA and 4-CBA under anaerobic conditions, whereas the former two readily degraded both compounds under aerobic conditions. Accumulation of any intermediary metabolite was not observed during growth as well as reaction with resting cells under highly aerated conditions. However, on modification of reaction conditions, 3-chlorocatechol (3-CC) and 4-chlorocatechol (4-CC) accumulated in 3-CBA and 4-CBA flasks, respectively. Fairly high titres of pyrocatechase II (chlorocatechol 1,2-dioxygenase) activity were obtained in extracts of cells grown on 3-CBA and 4-CBA. Meta-pyrocatechase (catechol 2,3-dioxygenase) activity against 4-CC and catechol, but not against 3-CC, was also detected in low titres. Accumulation of small amounts of 2-chloro-5-hydroxy muconic semialdehyde, the *meta*-cleavage product of 4-CC, was detected in the medium, when 4-CBA concentration was 4 mM or greater, indicating the presence of a minor *meta*-pathway in strain 3mT. However, 3-CBA exclusively, and more than 99% of 4-CBA were degraded through the formation of the respective chlorocatechol, via a modified *ortho*-pathway. This defies the traditional view that the microbes that follow chlorocatechol pathways are not very good degraders of chlorobenzoates. 4-Hydroxybenzoate was readily (and 3-hydroxybenzoate to a lesser extent) degraded by the strain, through the formation of protocatechuate and gentisate, respectively, as intermediary dihydroxy metabolites.

Abbreviations: C12O: Catechol 1,2-dioxygenase; C23O: catechol 2,3-dioxygenase, CFE: Cell-free extract; 3-CBA: 3-chlorobenzoate; 4-CBA: 4-chlorobenzoate; 3-CC: 3-chlorocatechol; 4-CC: 4-chlorocatechol; 3-HBA: 3-hydroxybenzoate; 4-HBA: 4-hydroxybenzoate; CHMS: Chlorohydroxy muconic semialdehyde; PCA: protocatechuic acid.

Introduction

Recently, we isolated a bacterial strain, *Pseudomonas Aeruginosa* 3mT, that is capable of degrading high concentrations of 3-chlorobenzoate (3-CBA, up to 8 g l^{-1}) and 4-chlorobenzoate (4-CBA, up to 12 g l^{-1}) (Ajithkumar 1998). We also showed that this strain can bioremediate soils contaminated with both the chlorobenzoates, thus helping to protect germination of tomato seeds (Ajithkumar et al. 1998). Because strain 3mT can degrade very high concentrations of chlorobenzoates, we want to understand the pathways it uses for chlorobenzoate biodegradation. In this paper, we describe the results of biochemical studies carried out to delineate the initial steps in chlorobenzoate biodegradation by strain 3mT. In the remainder

of this section, we review the several biochemical options known for initial chlorobenzoate attack.

Degradation of chlorobenzoates and other chloroaromatics by microorganisms generally occurs through the formation of chlorocatechols as central intermediates which are further degraded via the modified *ortho*-pathway (Reineke & Knackmuss 1988; Choudhry & Chapalamadugu 1991; Schlomann 1994). Chlorobenzoate is converted initially to chlorobenzoate dihydrodiol catalysed by a dioxygenase by inserting dioxygen to the aromatic ring, which then is converted to chlorocatechol by a dihydrodiol dehydrogenase. The chlorocatechol degradative pathway involves the activities of chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, dienelactone hydrolase and maleylacetate reductase (Vollmer et al. 1994; Hoier et al. 1994; Solianikova et al. 1995; Vollmer & Schlomann 1995; Fetzner 1998). The product formed, 3-oxoadipate, enters the TCA cycle. Chloromuconate cycloisomerase catalyses the chloride elimination Alternative to the generally observed intradiol cleavage of chlorocatechol, extradiol cleavage by catechol 2,3-dioxygenase (C230) of the *meta*-pathway has, occasionally, been observed in microorganisms (Higson & Focht 1992; Hollender et al. 1994; Arensdort & Focht 1995). However, further degradation of the ring cleavage product of 4-chlorocatechol (4-CC) seems to be a slow process, as these organisms grow very slowly on these substrates. Degradation of chloroaromatics via *meta*-pathway, when the intermediate is 3-chlorocatechol (3-CC) has, hitherto, been considered impossible, since 3-CC adversely affects C230, either inhibiting reversibly, acting as a chelating agent (Klecka & Gibson 1981) or causing an irreversible inhibition by the ring cleavage product, the reactive acylchloride (Bartels et al. 1984). However, recent reports indicate that productive degradation of 3-CC through *meta*-cleavage pathway is possible. A bacterial strain *P. putida* GJ31 that can degrade chlorobenzene via 3-CC using *meta*-pathway, without any apparent toxic effect, has been reported by Mans et al. (1997). They also have purified and characterised a novel chlorocatechol 2,3-dioxygenase from this strain (Kaschabek et al. 1998). Microorganisms that follow a chlorocatechol pathway can generally degrade only low concentrations of chlorobenzoates, irrespective of whether the ring cleavage is effected through *ortho*- or *meta*-mode (Reineke & Knackmuss 1988; Chaudhry & Chapalamadugu 1991; Schlomann 1994).

In several other bacteria, removal of chlorine substituents from chloroaromatics, at an early step of degradation, has been reported. These organisms have, generally, been shown to be capable of degrading fairly high concentrations of chloroaromatics (Shimao et al. 1989; Kobayashi et al. 1997). First experimental evidence for the direct removal of chlorine from the aromatic ring was presented by Johnston et al. (1972) in a *Pseudomonas* sp. growing on 3-CBA. This strain dehalogenates 3-CBA to form 3-hydroxybenzoic acid (3-HBA) which is then hydroxylated to gentisic acid by 3-HBA 6-hydroxylase. Dechlorination of a chlorobenzoate at the first step may be effected by an oxygenase or a hydrolytic dehalogenase (Hardman 1991; Janssen et al. 1994; Fetzner 1998). A dioxygenase-mediated dehalogenation has been observed in the conversion of 2-CBA to catechol by *Pseudomonas* sp. 2CBS (Fetzner et al. 1992). A two component 2-halobenzoate 1,2-dioxygenase that preferentially oxidizes 2-CBA has been purified from this strain. A three-component dioxygenase that *ortho*-dechlorinates 2-CBA as well as 2,4-dichlorobenzoate (2,4-DCBA) has been purified from *P. aeruginosa* strain 142 (Romanov & Hausinger 1994). A chlorobenzoate dioxygenase which oxidises 3-CBA at 3,4 or 4,5 positions has been reported in *Alcaligenes* sp. strain BR30 (Nakatsu & Wyndham 1993). All these enzymes have been proposed to catalyse the formation of *cis*-diols, which spontaneously rearomatise, with a concomitant release of chloride, forming a catechol (Fetzner 1998).

However, in a majority of the reported cases, the initial chloride elimination from chlorobenzoate, particularly 4-CBA, occurs through a hydrolytic reaction. The enzymes for this are present in a number of bacterial strains belonging to the genera *Pseudomonas, Arthrobacter, Acinetobacter, Alcaligenes, Nocardia* and *Corynebacterium* (Hardman 1991; Dunaway-Mariano & Babbitt 1994; Janssen et al. 1994; Fetzner 1998). Conversion of 4- CBA to 4-hydroxybenzoate (4-HBA) requires three enzymes, 4-chlorobenzoate coenzyme A ligase, 4-chlorobenzoyl-CoA dehalogenase and 4-hydroxybenzoyl-CoA thioesterase. The hydroxyl oxygen is derived from water. The dehalogenation occurs only after the activation of the substrate to its CoA derivative, in the presence of ATP. Recently, Kobayashi et al. (1997), for the first time, have shown that hydrolytic dehalogenation of 4-CBA by intact cells of *Acinobacter* strain St-1 can occur, even under anaerobic conditions. 4-HBA is formed

and then hydroxylated by 4-HBA-3-hydroxylase to form protocatechuic acid (PCA).

Ring cleavage of the dihydroxy compounds formed from 3-CBA or 4-CBA can occur through either an *ortho*- or a *meta*-mode. Gentisate is cleaved by the action of gentisate 1,2-dioxygenase to form maleylpyruvic acid. PCA is cleaved either by the *ortho*-mode by PCA 3,4-dioxygenase to form 3-carboxy-*cis*-*cis*-muconic acid or by *meta*-mode to form 2-hydroxy-4-carboxy muconic semialdehyde, by the action of PCA 4,5-dioxygenase (Fujisawa 1970; Ono et al. 1970; Harayama et al. 1992). Another extra-diol cleavage of PCA to form 5-carboxy-2-hydroxy muconic semialdehyde, effected by PCA 2,3-dioxygenase, has been reported by Crawford (1975).

Materials and methods

Chemicals

3-CBA, 4-CBA, 4-HBA and PCA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3-HBA, gentisate and catechol were from Aldrich Chemical Co. Inc. (Milkwaukee, Wis, USA) and 3-CC and 4-CC were purchased from Helix Corp (Vancouver, Canada). Sodium benzoate and succinic acid were procured from Ranbaxy Fine Chemicals Ltd. (S.A.S. Nagar, India). All other chemicals used in this study were of Analytical Reagent grade and were procured from standard companies.

Organism and culture conditions

P. aeruginosa 3mT used in this study was a laboratory isolate (Babu et al. 1995; Ajithkumar 1998; Ajithkumar et al. 1998). The culture was grown in a mineral salts medium containing either 3-CBA or 4-CBA as a carbon source, as described previously (Babu et al. 1995; Ajithkumar et al. 1998). The culture flasks were incubated on a rotary shaker (150 rev. min^{-1}) at 30 $\pm$ 1 °C.

Studies with resting cells, permeabilized cells and cell-free extracts

Cells were grown in a mineral-salts medium containing 3-CBA, 4-CBA, sodium benzoate or succinic acid (each 1 g l^{-1}), as carbon source and were harvested at mid-exponential phase of growth, by centrifugation at 8000 $\times$ *g* for 15 min at 4 °C. The cells were washed with 50 mM phosphate buffer (pH 7.0) and were suspended in the same buffer containing 5 mM dithiothreitol (DDT). Reactions were carried out by incubating the reaction mixture containing the cells and the required substrate, at 30 °C on a rotary shaker (150 rev. min^{-1}) both under aerobic and anaerobic conditions. Cell concentrations in the reaction mixture was adjusted to an OD_{550} of 1.0, unless otherwise stated.

Permeabilised cells were prepared, according to the optimized conditions, as follows: The cell biomass, grown as above, was washed twice with 100 mM sodium phosphate buffer (pH 7.0) and suspended in 10 ml of the same buffer containing cetyl trimethyl ammonium bromide (CTAB). The concentration of CTAB was adjusted to 10 mg g^{-1} wet cells. The cells were kept in the solution for 20 min at 8 °C and then pelleted by centrifugation at 10, 000 $\times$ *g* for 10 min. The cells were washed twice with phosphate buffer and used immediately for the metabolic studies, as above.

For making cell-free extracts (CFEs), thick suspensions (75 mg ml^{-1}, dry wt.) of cells, grown as above, were made either in 50 mM phosphate buffer (pH 7.5) or in 50 mM Tris HCl (pH 9.0). The cells were disrupted by ultrasonication at 10 KHz for 10 min with intermittent bursts of 30 seconds using Labsonic 2000 sonicator (B. Braun, Germany). Cell debris was removed by centrifugation at 30, 000 $\times$ *g* for 20 min. All operations were carried out at 4 °C. All the assays with these crude enzymes were carried out immediately.

Anaerobic experiments with resting cells or CFEs were performed in 10 ml air-tight vials containing phosphate buffer (50 mM, pH 7.0) and 5 mM DTT. The buffer was boiled for 30 min under nitrogen gas, injected into the vials and sealed. After cooling the vials to 30 °C, the cell suspension was injected, followed by the substrate solution. Oxygen-free nitrogen was used to flush the cell suspensions and substrate solution during the injection. The total volume of reaction mixture was 5 ml. To stop the reaction at different intervals, the vials were heated to 70 °C for 10 min. The reaction mixture was then analysed for residual substrate, release of chloride (Cl^-), and for the presence of metabolites, if any.

Assay of enzymes

Dehalogenase activity in CFE was determined by the method described by Marks et al. (1984a) using 3-CBA or 4-CBA as substrate. Residual substrate and

Cl^- release were determined, after the reaction was stopped.

Pyrocatechase (catechol 1,2-dioxygenase EC 1.13.1.1) was assayed spectrophotometrically according to the method of Nakazawa & Nakazawa (1970) based on the rate of formation of *cis,cis*-muconic acid or its derivatives from catechol or chlorocatechols. In a final volume of 3.0 ml of reaction mixture, 0.06 ml of substrate solution (0.01 M catechol or 0.5–1.0 μmol 3-chloro or 4-chlorocatechol) was incorporated. To calculate the concentrations of ring fission products from values of A_{260}, the molar extinction coefficients of substituted muconates, described by Dorn & Knackmuss (1978b) were used.

Metapyrocatechase (catechol 2,3-dioxygenase, EC 1.13.1.2) activity was determined by the method of Nozaki (1970) by measuring the increase in the OD at 375 nm caused by the formation of the reaction product, 2-hydroxy muconic semialdehyde or its chlorinated derivative.

Determination of chlorobenzoates and metabolites

Residual chlorobenzoates or its intermediary metabolites, in the culture supernatants, were determined by reverse phase high performance liquid chromatography (HPLC) (Shimadzu LC-6A, Japan), with a C18 column (150 $\times$ 4.6 mm). The mobile phase used was either methanol-water-acetic acid (40 : 60 : 1 v/v) or a gradient of methanol and water containing 1% acetic acid, for the separation of 3-CBA and 4-CBA from a mixture. The flow rate was 1 ml min^{-1} and detection was by a variable UV-absorbance detector. The metabolites were detected at 254 nm, 3-CBA and 4-CBA were detected at 235 nm, catechols at 275 nm, PCA at 290 nm and gentisic acid at 320 nm.

Protein estimation

Protein in CFE was estimated by the method of Lowry et al. (1951) using BSA as the standard.

Determination of inorganic chloride

Cl^- was estimated by a slightly modified procedure of Bergmann & Sanik (1957) as described earlier (Babu et al. 1995).

Estimation of catechols

Catechols were quantitatively determined by the method of Arnow (1937).

Isolation of intermediary metabolites

The dihydroxy derivatives from the culture supernatants and resting cell suspensions were isolated as follows: After removing the cells by centrifugation the supernatant was acidified by HCl to pH 2.0 and was extracted repeatedly, with equal volumes of ethyl acetate, till the broth showed negative to Arnow's test. The pooled extract was evaporated to 5 ml and dried over anhydrous sodium sulphate. This was analysed by thin layer chromatography (TLC) on silica gel G and also by HPLC, UV and IR spectroscopy, after partial purification by preparative TLC. A part of the extract was acetylated, using a mixture of acetic anhydride and pyridine (2 : 1). The acetylated sample was loaded onto a preparative TLC plate and developed in benzene-toluene- acetic acid (2 : 2 : 1). The spots were detected either by exposing to iodine vapour or under UV-illumination. For isolation of the metabolites from TLC, the compound band was scraped off and extracted twice with warm ethyl acetate. This extract was concentrated and used for UV and IR spectroscopy. UV-spectra of the isolated compounds were recorded by Shimadzu UV-160A (Japan) spectrophotometer. IR-spectra of the isolated metabolites were recorded either as KBr pellet or in nujol using a FT-IR spectrophotometer (IFS-25, Bruker, Germany).

Results and discussion

The initial reaction?

Cells grown on 3-CBA or 4-CBA rapidly metabolised the dihydroxy benzene derivatives, PCA, gentisic acid and catechol. They also metabolised 4-HBA efficiently, but not 3-HBA so well. As degradation of 4-CBA through the formation of 4-HBA, and that too at high concentrations, by bacteria is well established (Ruisinger et al. 1976; Klages & Lingens 1979; Zaitsev & Karasevich 1981; Marks et al. 1984a,b; Van den Tweel et al. 1986; Adriaens et al. 1989; Shimao et al. 1989; Tobita & Iyobe 1992; Kobayashi et al. 1997), 4-HBA was placed as a possible intermediate in the catabolism of 4-CBA by the strain 3mT. This was tested with resting cells of the strain 3mT, both under aerobic and anaerobic conditions. 3-CBA was also similarly tested, though the strain has not shown very good catabolism of 3-HBA. Both 3-CBA and 4-CBA were degraded well by resting cells, grown on either 3-CBA or 4-CBA, under aerobic conditions

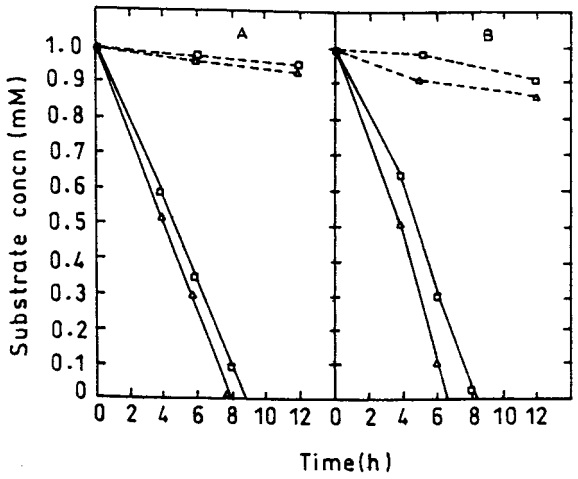

Figure 1. Degradation of 3-CBA (A) and 4-CBA (B) by intact resting cells (-□-) and permeabilised cells (-△-) of *Pseudomonas aeruginosa* strain 3mT under aerobic (solid lines) and anaerobic (broken lines) conditions. Substrate concentration was 1 mM. Other details of the experiment were as given in the text.

(Figure 1). Stoichiometric amounts of Cl^- were released from both the substrates (not shown in the figure). However, no degradation or Cl^- release was obtained under anaerobic conditions even after 12 h incubation (Figure 1). HPLC analysis confirmed that substrates (3-CBA and 4-CBA) were not degraded at all under anaerobic conditions.

Failure to dehalogenate 4-CBA and 3-CBA by resting cells under anaerobic conditions alone cannot rule out the possibility of 4-HBA and 3-HBA as intermediates, respectively, as there is a possibility of an energy dependent transport barrier in the cells. Hence, CFEs were used as the enzyme sources. However, both under aerobic and anaerobic conditions, they failed to dechlorinate both 3-CBA and 4-CBA. This would be due to the inactivation of the enzyme in question during the preparation of CFEs as observed by Van den Tweel et al. (1986, 1987) or may be due to the requirement for co-factors such as CoA or ATP for the reaction, as reported by several other workers (Loffler et al. 1991; Scholten et al. 1991; Copley & Crooks 1992). Kobayashi et al. (1997) also have observed the failure of the cell extracts to dehalogenate 4-CBA. Others, however, have shown the dehalogenating activity, in cell extracts prepared from 4-CBA-degraders (Klages & Lingens 1980; Marks et al. 1984a; Muller et al. 1984; Thiele et al. 1987; Shimao et al. 1989). As the enzyme in CFEs was unstable, permeabilized cells were used for dehalogenase assay under anaerobic conditions. No dechlorination of 4-CBA or 3-CBA was obtained, whereas under aerobic conditions CTAB-permeabilised cells showed faster conversion of both the substrates (Figure 1). Failure to dehalogenate the substrates under anaerobic conditions indicated the clear possibility of the involvement of an oxygenase in the initial reaction. Oxydative dehalogenation of chloroaromatics have been reported (Harayama et al. 1992; Fetzner & Lingens 1994; Butler & Mason 1997; Fetzner 1998). A two-component 2-halobenzoate 1,2-dioxygenase-mediated dehalogenation has been shown in the conversion of 2-CBA by *P. cepacia* 2CBS (Fetzner et al. 1992). *Ortho*-dechlorination of both 2-CBA and 2,4-DCBA by a 3-component dioxygenase from *P. aeruginosa* strain 142 also has been reported (Romanov & Hausinger 1994). A chlorobenzoate dioxygenase having poor regioselectivity, present in *Alcaligenes* sp. strain BR30, oxidizes 3-CBA at the 3.4 or 4.5 positions (Nakatsu & Wyndham 1993). However, in the majority of cases of aerobic degradation of chlorbenzoate through modified *ortho*-pathway, the ring hydroxylation by dioxygenases followed by dehydrogenation, results in the formation of halocatechols as the key intermediates.

Use of metabolic inhibitors

Tiron, α-α'-dipyridyl and EDTA, being metal-chelators, are known to inhibit ring cleaving dioxygenases and hence, are useful in specifically inhibiting the ring cleavage reaction, resulting in the accumulation of dihydroxy benzene derivatives, providing the first enzyme in the pathway is a hydrolytic dehalogenase. If it is so, PCA and gentisate are expected to accumulate in the reaction mixture, containing 4-CBA and 3-CBA, respectively as substrates. As no clear evidence was obtained from the previous experiment, as to what reaction occurs in the first step, alternatively this approach was made. The inhibitors were added to the resting cell suspensions of the strain 3mT at 3 concentrations, viz., 1, 5 and 10 mM and incubated for 30 min. The substrate, 3-CBA or 4-CBA, was then added to obtain 2 mM concentration and incubated on a rotary shaker (150 rev min^{-1}). The results are presented in Table 1. α,α'-Dipyridyl inhibited the metabolism of chlorobenzoates even at 1 mM level whereas Tiron had no effect. With EDTA, a decreased degradation of chlorobenzoates at 5 mM and complete inhibition at 10 mM was observed. No intermediary metabolites accumulated in the reaction mixture. These results also indicated that the first enzyme is not a hydrolytic dehalogenase, but an oxygenase. All the

ring hydroxylating dioxygenases are non-haem iron multi-component enzymes (2 or 3 components) requiring, for their activity, co-factors such as NADH or NADPH and, in the majority of cases, iron in addition to oxygen. All of them contain iron sulphur clusters for transfer of electrons to the terminal oxygenase (Butler & Mason 1992; Harayama et al. 1992; Mason & Cammack 1992).

Adriaens et al. (1989) and Tobita & Iyobe (1992) have shown the hydrolytic dehalogenation of 4-CBA by *Acinetobacter* strains. *Alcaligenes denitrificans* NTB-1 (now called coryneform bacterium strain NTB-1) also dehalogenated 4-CBA to 4-HBA, but the product accumulation was observed under low and controlled oxygen concentration (Van den Tweel et al. 1986). No assimilation and dehalogenation of 4-CBA occurred in this bacterium under anaerobic conditions unless an alternative electron acceptor such as nitrite was present (Groenewegen et al. 1992). Marks et al. (1984a,b) reported the dechlorination of 4-CBA by the cell extract of *Arthrobacter* sp. strain TM-1 under anaerobic conditions. Dehalogenation and formation of 4-HBA in this case was inhibited by dissolved oxygen and was stimulated by manganese. In *Arthrobacter* sp. strain SB8, oxygen was needed for 4-CBA dehalogenation by resting cells. But, with CFE, oxygen was inhibitory (Shimao et al. 1989). Hydrolytic dehalogenation of 4-CBA to 4-HBA by intact cells (resting cell) of *Acinetobacter* sp. ST-1, both under aerobic and anaerobic conditions, was claimed for the first time by Kobayashi et al. (1997). The dehalogenating enzyme is not an oxygenase but the halide hydrolase complex which catalyses the replacement of —Cl with —OH, deriving the required oxygen from water. Loffler et al. (1991) have shown the involvement of a coenzyme A-ATP-dependent dehalogenation of 4-CBA by the cells of *Pseudomonas* sp. CBS3.

In the present study, the inability of resting cells, CFE and permeabilized cells to dehalogenate 3-CBA and 4-CBA under anaerobic conditions is indicative of the absence of a dehalogenase in strain 3mT, that dechlorinates these compounds hydrolytically (i.e., using oxygen from water). The inhibitors, known to inactivate ring cleaving dioxygenases through metal (iron) chelation, inhibited the first reaction itself, further suggesting the possibility of the first enzyme being a (di)oxygenase. Hence, it can be inferred that a chlorobenzoate dioxygenase is involved in the hydroxylation of 4-CBA and 3-CBA.

Isolation and identification of intermediary metabolites

During the active growth of the strain 3mT on 3-CBA or 4-CBA, no accumulation of any metabolite was detected. Samples of culture supernatants, drawn during exponential and stationary phases of growth, when extracted with ethyl acetate, failed to give sufficient quantities of any of the intermediary metabolites on TLC. However, when 5 mM 4-CBA was incubated with resting cells for 1 h at 30 °C with shaking and then for 8 h at 8 °C without shaking (to reduce the metabolic rate), a dihydroxy compound, as detected by Arnow's test, accumulated in the medium. This was extracted with ethyl acetate and purified by TLC. UV-Spectral properties of this compound compared very well with standard 4-CC (Figure 2B). Solutions of the isolated metabolite and standard 4-CC in 0.1 M HCl, distilled water and 0.1 M NaOH showed absorption maxima at 294, 284 and 311 nm, respectively. TLC analysis of this compound also revealed similarity with 4-CC (Table 2). The identity of this compound as 4-CC was then confirmed by IR-spectroscopy (Figure 3). Similarly, resting cells, with 3-CBA as the substrate, yielded a dihydroxy compound, with spectral properties similar to 3-CC (Figure 2A). Acidic (0.1 M HCl) and neutral (distilled water) solutions of both the isolated metabolite and standard 3-CC showed absorption peaks at 290 and 274 nm, respectively. In 0.1 M NaOH both of them showed absorption maxima at 274 and 317 nm. Isolation of 3-CC from the reaction mixture often resulted in poor yield, due to its instability and high rates of autooxidation and polymerisation and hence, could not be purified for IR-spectral analysis. The UV-spectrum (Figure 2A) and R_f values on TLC (Table 2), however, strongly suggested the formation of 3-CC from 3-CBA. 4-CC was not detected in the reaction mixture of 3-CBA and *vice-versa* 3-CC was not obtained from 4-CBA.

Studies with a mutant of P. aeruginosa *3mT*

While attempting to cure the strain 3mT of a native plasmid by ethidium bromide treatment, a mutant, which had lost the ability to utilise 3-CBA and 4-CBA as carbon sources, was isolated. The mutation, however, was not due to the loss of the R' plasmid, as plasmid was intact in the mutant too (the data will be published elsehwere). When benzoate-grown cells of this mutant were incubated with 3-CBA or 4-CBA dihydroxy compounds, which were identified as 3-CC and 4-CC, respectively, were obtained in substantial

Table 1. Effect of different dioxygenase inhibitors on the metabolism of 3-chloro- and 4-chlorobenzoic acids by resting cells of *P. aeruginosa* 3mT.

Inhibitor		Disappearance[a] (%) of	
Compound	Concentration (%)	3-CBA	4-CBA
Control	Nil	100	100
EDTA	1.0	100	100
	5.0	81	83
	10.0	Nil	Nil
Tiron	1.0	84	81
	5.0	80	82
	10.0	78	81
α-α'-Dipyridyl	1.0	Nil	Nil
	5.0	Nil	Nil
	10.0	Nil	Nil

[a] Disappearance after an incubation period of 5 h as determined by HPLC.

Table 2. R_f values on TLC of the dihydroxycompounds isolated from the reaction mixture containing resting cells of the strain 3mT and 3-CBA or 4-CBA.

Solvent system	R_f (cm)			
	Metabolite from 3-CBA	3-Chlorocatechol	Metabolite from 4-CBA	4-Chlorocatechol
A	0.79	0.78	0.75	0.74
B	0.50	0.49	0.26	0.27

A: Benzene-toluene-acetic acid (2 : 2 : 1).
B: Chloroform.

amounts. Further degradation of chlorocatechol was not effected by the mutant strain. This finding also indicates that strain 3mT follows chlorocatechol pathways, for the degradation of 3-CBA and 4-CBA, and that the first enzyme of the reaction is a dioxygenase.

A minor meta-*pathway*

When 4-CBA was used at higher concentrations, viz., 4 mM or greater, the growth medium turned slightly yellowish, during the late exponential phase. This coloured compound showed an absorption maximum at 378 nm, and apparently was not by *P. aeruginosa* pigments. On acidification the colour disappeared, and could be restored by addition of alkali. Culture filtrate was positive for aldehyde group test. These characters are close to 2-chloro-5-hydroxy muconic semialdehyde (CHMS), the *meta*-cleavage product of 4-chlorocatechol (4-CC) (Hartmann et al. 1979). When 4-CBA, at concentrations of 4–10 mM, was incubated with resting cells, accumulation of CHMS, proportional to the 4-CBA concentration, was observed. A maximum level of about 35 μM of CHMS was obtained, when the substrate concentration was 10 mM. This is about 0.35% of the theoretical concentrations of CHMS expected from 10 mM 4-CBA. That means, it is a very minor *meta*-pathway (less than 1%) that is operative in the strain 3mT, the major pathway being a modified *ortho*-pathway. No yellow coloured compound was detected in the culture filtrates when 3-CBA was the substrate. But, when the concentration of 3-CBA was above 8 g l^{-1} the medium turned deep brown or black, due to the accumulation, autooxidation and polymerization of 3-CC. Degradation of 4-CBA, an intermediate in the metabolism of 4-chlorobiphenyl by *P. cepacia* P166, through *meta*-cleavage of 4-CC was reported by Arensdorf and Focht (1995). Higson and Focht (1992) have shown utilisation of 3-chloro-2-methyl benzoic acid by *P. cepacia* MB2 through *meta*-fission pathway. *Comamonas testosteroni* JH5 is capable of degrading both chloro- and methylphenol, through *meta*-fission pathway (Hollender et al. 1994). Hartmann et al. (1989) have reported 4-CC to be a good inducer and substrate

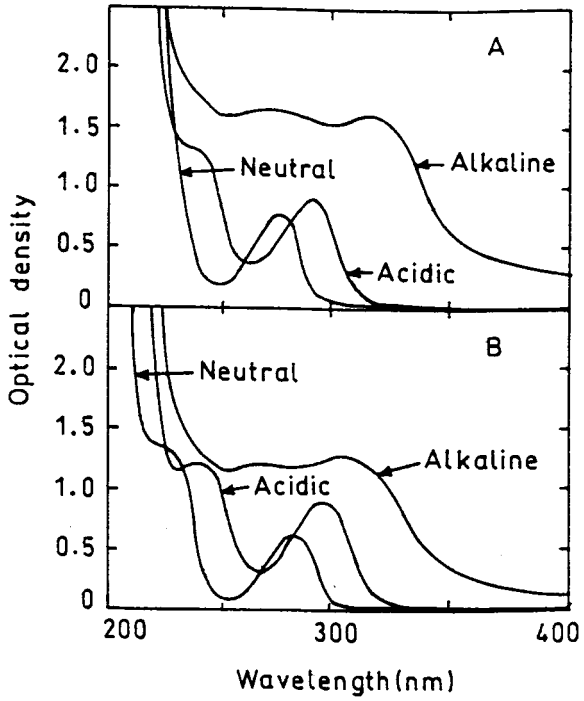

Figure 2. Spectral properties of the intermediary metabolites 3-CC (A) and 4-CC (B) isolated from the reaction mixture of resting cells of *Pseudomonas aeruginosa* 3mT and 3-CBA and 4-CBA, respectively. Experimental details are given in the text.

for a C230 in an *in vivo* constructed pseudomonad. C230 can convert 4-CC at respectable rates. But, further degradation of the ring cleavage product of 4-CC seems to be a slow process (Mars et al. 1997; Kaschabek et al. 1998). In the present case, probably, the metacleaving C230 is very inefficient as compared to chlorocatechol 1,2-dioxygenase. That could be the reason why only a minor portion of 4-CBA was processed through *meta*-pathway.

Enzymological studies

Determination of various enzyme activities was done with CFEs prepared from cells grown on 3-CBA, 4-CBA, benzoate or succinate. As already mentioned, the first enzyme of the 3-CBA/4-CBA pathway, the dioxygenase, was not stable in CFEs and, hence, no activity was detected. Similarly, 4-HBA monoxygenase was also not detected in CFEs, though the resting cells readily metabolised 4-HBA. This may also be because these enzymes were inactivated during the preparation of CFE. As discussed earlier, oxygenases require various co-factors, which might be lost during the preparation of the crude enzymes, or/and the enzyme itself may be getting inactivated. Varying levels of the ring-cleaving enzyme activities were detected in CFEs of cells grown on 3-CBA, 4-CBA and benzoate whereas no activity was obtained in cells grown on succinate (Table 3). Chlorocatechols were effectively cleaved by CFEs of cells grown on 3-CBA and 4-CBA, and the C12O activity on 3-CC and 4-CC was very low in benzoate-grown cells. This suggests the occurrence of two types of pyrocatechases, one induced by both benzoate and chlorobenzoates and another induced only by chlorobenzoates. *Meta*-cleavage (C23O) activity against 4-CC, though in low titres, was detected in CFEs of cells grown on 3-CBA and 4-CBA, the activity being higher in the latter (Table 3). Though the CFE of benzoate-grown cells showed a little C230 activity against catechol, it failed to cleave 4-CC. No C23O activity against 3-CC was detected in any CFEs. Chlorobenzoates and other chloroaromatics are, generally, degraded via the formation of chlorocatechols as central intermediates, which are further degraded through the modified *ortho*-pathway (Reineke & Knackmuss 1988; Schlomann 1994; Vollmer & Schlomann 1995; Solianikova et al. 1995). However, a few bacterial strains capable of cleaving 4-CC, formed during the catabolism of monochlorobiphenyl, 4-CBA, 3-chloro-4-methyl benzoic acid and chlorophenol, by *meta*-fission enzyme have been reported, though the further degradation of the cleaved product is rather slow (Higson & Focht 1992; Hollender et al. 1994; Arensdorf 1995). In the present case too, an extradiol ring-cleaving enzyme induced by both 3-CBA and 4-CBA was detected in low titres. Accumulation of the ring-cleavage product also was observed, either because of its slow degradation or due to the complete absence of the enzymes required for further catabolism. No *meta*-cleaving activity against 3-CC was observed in *P. aeruginosa* 3mT. This could be due to the suicide inactivation of the enzyme through the formation of a highly reactive acylchloride from 3-CC (Bartels et al. 1984). However, a bacterial strain, *P. putida* GJ31 that degrades chlorobenzene using *meta*-pathway without any apparent toxic effects has been reported (Mars et al. 1997). An unusual *meta*-fission enzyme, C230 that cleaves 3-CC, formed from chlorobenzenes was isolated from this strain. The enzyme also dehalogenates 3-CC during ring-cleavage and forms 2-hydroxymuconate as the product (Kaschabek et al. 1998). Induction of both *ortho*- and *meta*-cleaving enzymes in a strain of *Burkholderia cepacia* (formerly *P. cepacia*) has been shown

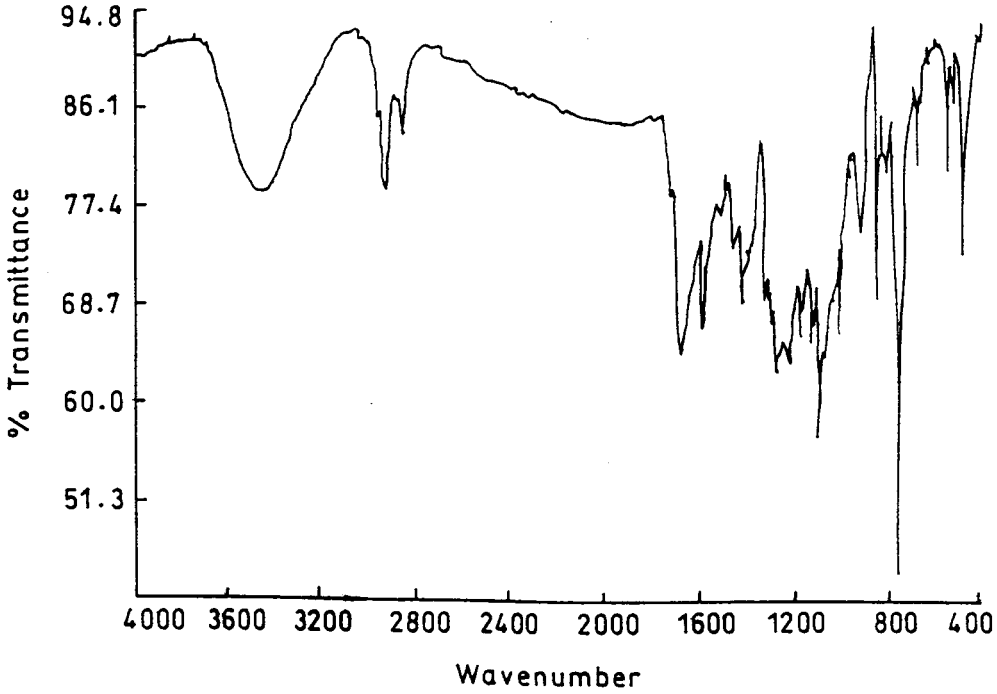

Figure 3. Infrared absorption spectrum of the metabolite, 4-chlorocatechol, isolated from resting cell suspension of *Pseudomonas aeruginosa* 3mT containing 4-chlorobenzoate as the substrate.

by Hamzah & Al-Baharna (1994), the ratio between C12O and C23O being 7 : 1.

Reaction with resting cells grown on 3-CBA, 4-CBA, benzoate or succinate, also threw some light on the inducibility of some of the degradative enzymes (Figure 4). When 3-CBA and 4-CBA were used as the assay substrates, no Cl^- release occurred with cells grown on succinate, and only low levels of Cl^- release was observed when benzoate-grown cells were used. Both 3-CBA and 4-CBA-induced cells, however, showed the release of copious amounts of Cl^-, 3-CBA-induced cells being more efficient. Accumulation of chlorocatechols (formed from 3-CBA/4-CBA) was observed when benzoate grown cells were used (data not given), whereas chlorocatechols did not accumulate in the reaction mixture when 3-CBA or 4-CBA grown cells were used. It is clear that the 3-CBA and 4-CBA degradative pathway(s) are inducible and are induced effectively by the substrates and also by benzoate, a substrate analogue, to a lesser extent (Table 3 and Figure 4). C12O of benzoate-grown cells showed only slight activity against 3-CC and 4-CC, whereas 3-CBA/4-CBA-grown cells showed much higher activity against chlorocatechols as well as catechol (Table 4). This points to the presence of isoenzymes for the metabolism of chlorobenzoates and benzoate. Benzoate-grown cells converted 3-CBA and 4-CBA efficiently to their respective chlorocatechols. This could, probably, be due to the induction of a broad spectrum ring-hydroxylating benzoate 1,2-dioxygenase (BO), which may be converting both benzoate and chlorobenzoates. With resting cells of 3mT, the efficiency of chlorobenzoate turnover was in the order (with respect to the inducing substrate) 3-CBA > 4-CBA > benzoate. This is similar to *Pseudomonas* WR912 (Hartmann et al. 1979) and TOL plasmid-coded enzyme of *P. putida*. TOL plasmid-coded toluene 1,2-dioxygenase was shown to be stereo-non-specific and considerably more active towards halogenated substrates. This enzyme has been purified, and a wide range of substituted aromatic compounds were found to be its substrates (Zeyer et al. 1985). *Pseudomonas* sp. B13, however, could utilise only 3-CBA, because of the narrow specificity of the chlorozenzoate 1,2-dioxygenase system (Dorn et al. 1974). The limited efficiency of the crude enzyme of benzoate-grown cells to cleave chlorocatechols (Table 3) strongly points to the presence of two types of pyrocatechases, one for catechol and the second for chlorocatechols. 3-CBA induced enzyme showed about 91% and 66% activity against 4-CC and 3-CC, and 4-CBA induced enzyme showed about 40% and 46% activity, respect-

Table 3. Specific activities of ring-cleaving enzymes in cell-free extracts of *Pseudomonas aeruginosa* strain 3mT grown on different substrates. Details of preparation of cell extracts and determination of enzyme activities were as given in the text. Values in parenthesis indicate the percentage relative activities of both C120 and C230 against different substrates taking the activity against catechol as 100%.

	Assay substrate	Specific activities (units/mg protein) when grown with			
		3-CBA	4-CBA	Benzoate	Succinate
Catechol-1,2-dioxygenase	Catechol	0.246	0.200	0.184	Nil
		(100)	(100)	(100)	
	3-Chlorocatechol	0.162	0.091	0.013	Nil
		(65.85)	(45.5)	(7.06)	
	4-Chlorocatechol	0.223	0.079	0.013	Nil
		(90.65)	(39.5)	(7.06)	
Catechol-2,3-dioxygenase	Catechol	0.004	0.006	0.002	Nil
			(100)	(100)	(100)
	3-Chlorocatechol	Nil	Nil	Nil	Nil
	4-Chlorocatechol	0.002	0.004	Nil	Nil
		(50)	(66.66)		

ively, whereas benzoate-induced enzyme showed only 7% activity towards both the substrates, when activity of each enzyme against catechol was taken as 100% (Table 3). This could be because of the broader substrate specificity of chlorocatechol 1,2-dioxygenase (pyrocatechase II) than the limited specificity of catechol 1,2-dioxygenase (pyrocatechase I) (Broderick & O'Halloran 1991; Van der Meer et al. 1991; Harayama et al. 1992; Schlomann 1994). Isoenzymes have been characterised in *Pseudomonas* sp. B13, and pyrocatechase I was shown to be highly specific for catechol, whereas pyrocatechase II showed higher activity towards chlorocatechols (Dorn & Knackmuss 1978a,b). Pyrocatechase I was inducible with benzoate and chlorobenzoate whereas pyrocatechase II was inducible with 3-CBA and 4-chlorophenol. But, in the case of *Pseudomonas* sp. WR912, benzoate could induce both the pyrocatechases (Hartmann et al. 1979). Strain 3mT resembles *Pseudomonas* sp. B13 in this respect, as a respectable amount of pyrocatechase II is induced only by chlorobenzoates.

The specific activities of pyrocatechase II in the CFE of strain 3mT grown on 3-CBA against 3-CC and 4-CC were 0.162 and 0.223 unit mg^{-1} protein, respectively. These high activities could be the reason why the strain 3mT can tolerate and efficiently degrade higher concentrations of 3-CBA (upto 8.0 g l^{-1}) and 4-CBA (upto 12.0 g l^{-1}) (Ajithkumar 1998). Strain 3mT is the first bacterium to be reported to have the ability to degrade such high concentrations of these substrates. Naturally, one would think that this strain may be eliminating Cl^- in the early stages,

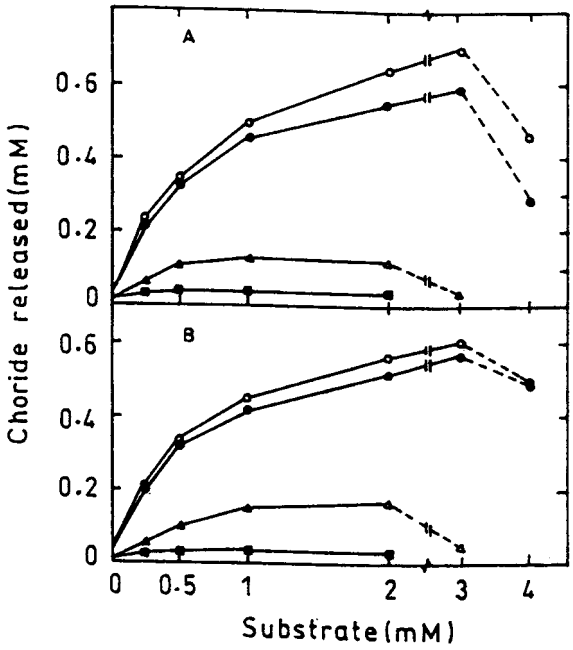

Figure 4. Dechlorination of 3-CBA (A) and 4-CBA (B) by resting cells of *Pseudomonas aeruginosa* 3mT grown on 3-CBA (-○-), 4-CBA (-●-), benzoate (-△-) or succinate (-■-). Experimental details are given in the text.

thus avoiding the toxicity of the common intermediate, chlorocatechol (Shimao et al. 1989; Kobayashi et al. 1997). *Arthrobacter* sp. strain SB8 of Shimao et al. (1989) can degrade upto 10 g l^{-1} of 4-CBA, through the dechlorination of the substrate is the first step itself. All the other strains that follow chlorocatechol

Table 4. Chromatographic properties of dihydroxy metabolites isolated from the reaction mixture containing resting cells of strain 3mT and 3-hydroxy- or 4-hydroxybenzoic acid in the presence of α-α'-dipyridyl (1 mM).

	R_f (cm) on (TLC)	Retention time (min) in HPLC
Metabolite from 3-HBA reaction mixture	0.52	5.12
Gentisic acid (standard)	0.52	5.20
Metabolite from 4-HBA reaction mixture	0.40	4.50
Protocatechuic acid (standard)	0.41	4.60
3-HBA (standard)	0.57	6.40
4-HBA (standard)	0.57	6.30

The solvent system for TLC was benzene-toluent-acetic acid (2 : 2 : 1), and the compounds were detected by viewing under UV (320 nm) as well as by exposing to iodine vapour. For HPLC, the solvent system was methanol : water : acetic acid (40 : 59 : 1).

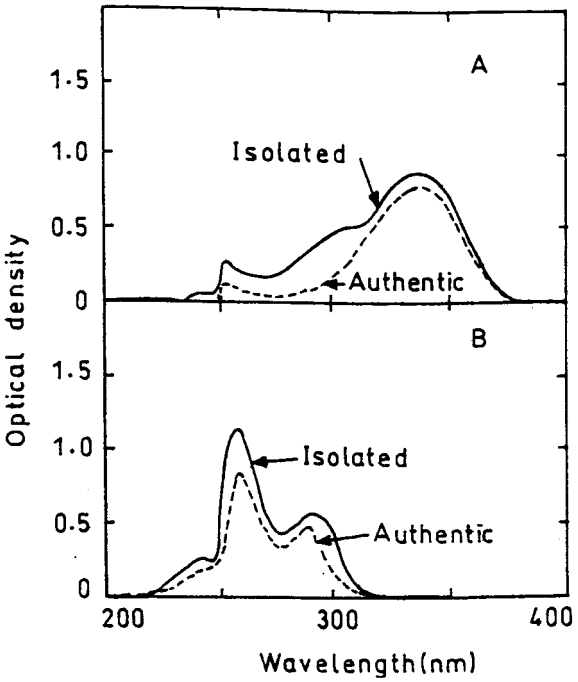

Figure 5. Spectral properties of metabolites formed from 3-hydroxygbenzoic acid (A) and from that of 4-hydroxybenzoic acid (B) by *Pseudomonas aeruginosa* 3mT. Details of the experiment are in text.

pathways for chlorobenzoate degradation can degrade only a maximum concentration of up to 2 g l^{-1} of the substrate (Reineke & Knackmuss 1988; Chaudhry & Chapalamadugu 1991; Ajithkumar 1998). But, the present finding proved otherwise.

Metabolism of hydroxybenzoates

4-HBA is generally metabolised through the formation of PCA (Shoun et al. 1983; Schreuder et al. 1990; Entsch et al. 1991; Eulberg et al. 1998) and 3-HBA through gentisic acid or PCA (Michalover and Ribbons 1973; Wang et al. 1987; Rajasekharan et al. 1990). The first enzyme responsible for conversion of 4-HBA or 3-HBA is a monooxygenase. Resting cells of strain 3mT grown on either 3-CBA or 4-CBA were incubated with 3-HBA and 4-HBA and the formation of metabolites were monitored. To thick suspension of the cells (OD_{550} = 5.0) in 25 ml of 50 mM phosphate buffer (pH 7.0), was added 25 mg of 3-HBA or 4-HBA and was incubated at 30 °C, with stirring. Formation of dihydroxy compounds was monitored at regular intervals. After 5 h, though the conversion of 4-HBA was complete (as determined by HPLC), no conversion product was detected in the reaction mixture. By the same time only 20% of 3-HBA was converted and here too no products were detected in the reaction mixture. However, when α-α'-dipyridyl was incorporated into the reaction mixture, dihydroxy compounds accumulated in both 3-HBA and 4-HBA flasks. HPLC analysis of the reaction mixtures of 3-HBA and 4-HBA revealed new compounds, having retention times of 5.2 and 4.5 min, respectively, which were similar to those of standard gentisate and PCA, respectively (Table 4). This compound was extracted and TLC was done. The R_f values of these compounds also agreed very well with those of standard compounds (Table 4). These compounds were purified by preparative TLC, and UV-spectra were taken. UV-spectral characters were similar to those of gentisate and PCA, respectively (Figure 5). Conversion of 4-HBA and 3-HBA to PCA and gentisate, respectively and their further metabolism to TCA-cycle intermediates by cells induced by 4-CBA and 3-CBA, is rather intriguing, particularly because the organism does not follow these pathways for degradation of 4-CBA and

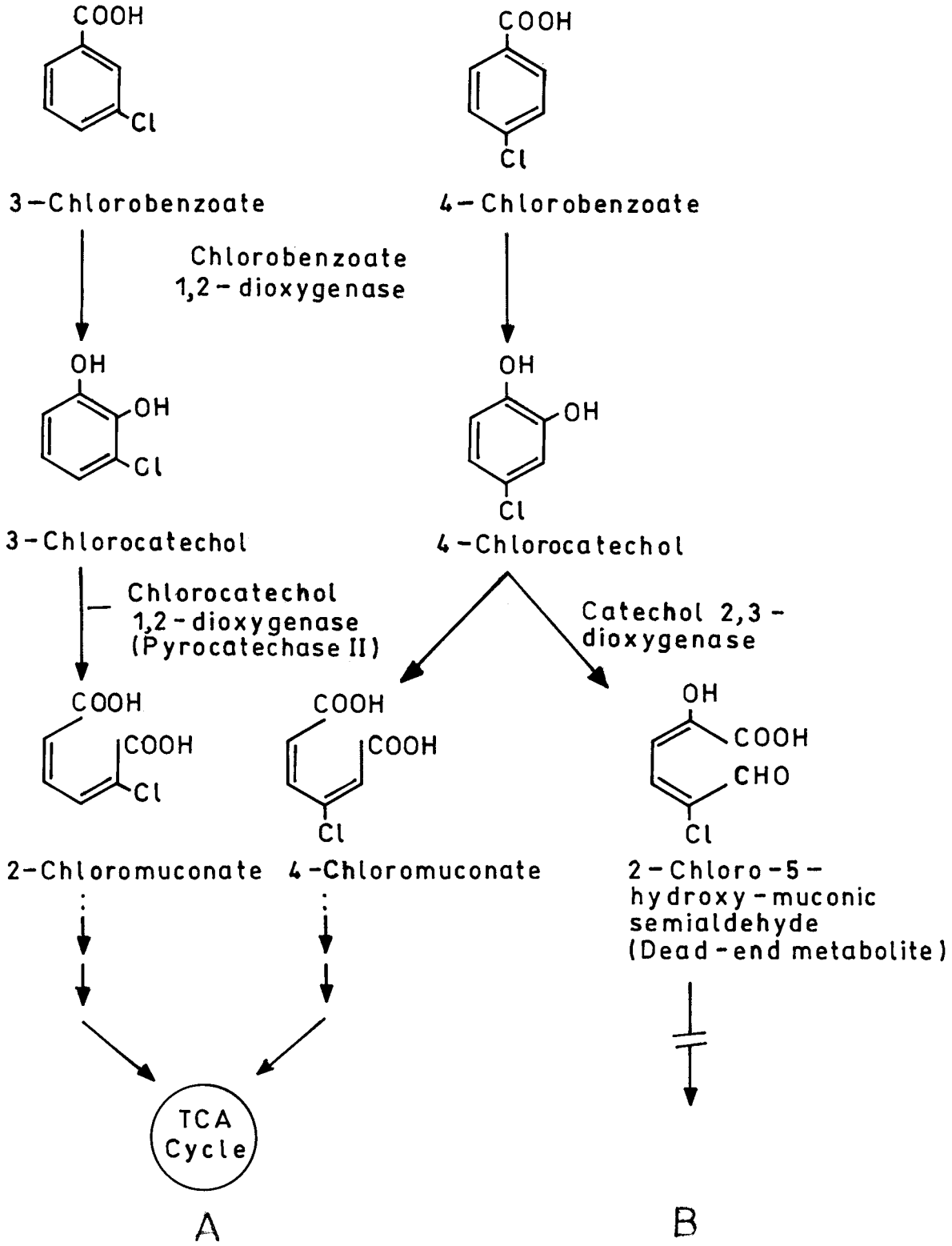

Figure 6. Proposed early steps of 3-chlorobenzoate and 4-chlorobenzoate catabolic pathways of *Pseudomonas aeruginosa* 3mT. (A) Major *ortho*-pathways (B) minor *meta*-pathway.

3-CBA. It is possible that 3-CBA and 4-CBA induce the synthesis of monooxygenase(s) with broad specificity. However, further detailed studies are required before any conclusion could be drawn.

In summary, the metabolic and enzymological studies clearly and unequivocally proved that 3-CBA and the major part of 4-CBA are degraded by *P. aeruginosa* 3mT via a modified *ortho*-pathway through the formation of 3-CC and 4-CC as intermediary metabolites, respectively. It was observed that the pathways are induced by 3-CBA, 4-CBA and to a lesser extent, by benzoate. Fairly high pyrocatechase II activities were observed in CFEs of cells grown on 3-CBA and 4-CBA. This could be the reason why the strain can degrade high concentrations of both the substrates effectively. This defies the "conventional wisdom" that more effective and faster degradation of chlorobenzoates can occur, if the chlorine is eliminated in the first step and that the degradation through chlorocatechol pathway(s) can occur only at low concentrations. It is, however, necessary to purify and characterise the pyrocatechese II of strain 3mT, to understand what makes this enzyme different from the earlier reported ones. A minor *meta*-pathway is also operative in the organism for the catabolism of 4-CBA (less than 1% of the total) and results in the accumulation of a dead-end metabolite, a chloro-hydroxy muconic semialdehyde. Based on these findings, pathways of 3-CBA and 4-CBA degradation are proposed (Figure 6).

Acknowledgment

The authors wish to thank Dr V. Prakash, Director, Dr Krishna Nand (Head, Food Microbiology), Central Food Technological Research Institute, Mysore, for their encouragement and permission for publishing the data. PVA also acknowledges with gratitude the financial support received from the CSIR (Govt. of India) in the form of a research fellowship.

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