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ORIGINAL PAPER

Biological degradation of 4-chlorobenzoic acid by a PCB-metabolizing bacterium through a pathway not involving (chloro)catechol

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Abstract *Cupriavidus* sp. strain SK-3, previously isolated on polychlorinated biphenyl mixtures, was found to aerobically utilize a wide spectrum of substituted aromatic compounds including 4-fluoro-, 4-chloro- and 4-bromobenzoic acids as a sole carbon and energy source. Other chlorobenzoic acid (CBA) congeners such as 2-, 3-, 2,3-, 2,5-, 3,4- and 3,5-CBA were all rapidly transformed to respective chlorocatechols (CCs). Under aerobic conditions, strain SK-3 grew readily on 4-CBA to a maximum concentration of 5 mM above which growth became impaired and yielded no biomass. Growth lagged significantly at concentrations above 3 mM, however chloride elimination was stoichiometric and generally mirrored growth and substrate consumption in all incubations. Experiments with resting cells, cell-free extracts and analysis of metabolite pools suggest that 4-CBA was metabolized in a reaction exclusively involving an initial hydrolytic dehalogenation yielding 4-hydroxybenzoic acid, which was then hydroxylated to protocatechuic acid (PCA) and subsequently metabolized via the β-ketoadipate pathway. When strain SK-3 was grown on 4-CBA, there was gratuitous induction of the catechol-1,2-dioxygenase and gentisate-1,2-dioxygenase pathways, even if both were not involved in the metabolism of the acid. While activities of the modified *ortho*- and *meta*-cleavage pathways were not detectable in all extracts, activity of PCA-3,4-dioxygenase was over ten-times higher than those of catechol-1,2- and gentisate-1,2-dioxygenases. Therefore, the only reason other congeners were not utilized for growth was the accumulation of CCs, suggesting a narrow spectrum of the activity of enzymes downstream of benzoate-1,2-dioxygenase, which exhibited affinity for a number of substituted analogs, and that the metabolic bottlenecks are either CCs or catabolites of the modified *ortho*-cleavage metabolic route.

Keywords Hydrolytic dehalogenation · Chlorobenzoic acid · Biodegradation · Protocatehuate-3,4-dioxygenase · Chlorocatechol

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Introduction

Owing to massive industrialization and other activities, toxic xenobiotic chemicals have become ubiquitous contaminants of various environmental matrices. The major environmental concern stems from recalcitrance to microbial and abiotic degradation, resulting in

persistence in the environment and accumulation in the food chain (Kunze et al. 2009). Approximately 2,500,000 contaminated sites were reported by the European Environmental Agency to be in urgent need of remediation as a consequence of the risks and hazards pose to human health and fragile ecosystems (EEA 2014). A similar trend (about 1322 sites) has been reported in the United States (USEPA 2015) and in Japan where a recent nuclear plant disaster rendered the immediate environment grossly contaminated with radioactive and allied chemicals. Environmental contamination problems are even more serious in developing countries where poor regulation is prevalent and improper disposal of chemicals continues unchecked. In Nigeria for instance, contaminated sites are ill-defined, pollution incidence is rarely reported, and the polluting companies are often rarely held accountable for their actions.

Among the xenobiotics requiring urgent cleanup, halogenated aromatic compounds, particularly polychlorinated biphenyls (PCBs) and chlorobenzoic acids (CBAs), constitute a unique and formidable class of pollutants (Wang et al. 2010). The recalcitrance and toxicity of these chemicals is a function of the number and respective position of chlorine substituents on the aromatic nucleus (Chaudhry and Chapalamadugu 1991). CBAs comprise a heterogenous group of organic contaminants whose presence in environmental systems is traceable to the widespread use of pesticides (Holtze et al. 2007) and partial metabolism of PCBs and allied chemicals (Adebusoye et al. 2007, 2008; Adebusoye and Mileto 2011). Toxicological investigations have implicated some CBAs as possible carcinogens, endocrine disruptors, and teratogens. These concerns therefore, have prioritized CBAs as major targets for cleanup of affected ecosystems.

It is evident from several studies that CBAs can be degraded by microorganisms, although only very few are known to utilize the acids as growth substrates (Adebusoye et al. 2008; Adebusoye and Mileto 2011). In addition, those organisms with the capacity to utilize CBAs more often than not, do not possess the requisite enzymatic machinery to degrade the parent compound (for example, PCBs), from which they were derived. Conversely, those organisms with capacity to metabolize the parent compounds often excrete CBAs as dead-end products (Adebusoye et al. 2007, 2008; Kim and Picardal 2000, 2001). Only two or three organisms, for example, are known to degrade PCBs to CBAs and ultimately mineralize the CBAs (Arensdorf and Focht 1994; Kim and Picadal 2000, 2001; Ilori et al. 2008).

Findings from various studies have revealed a number of pathways for the metabolism of CBAs depending on the position and degree of substitution as well as the type of microorganisms involved (Smith 1990; Mars et al. 1997; Adebusoye and Mileto 2011). In aerobic systems, the majority of organisms capable of growth on CBA congeners utilize relatively non-specific enzymes to activate the aromatic ring to chlorocatechols (CCs) as key intermediate products (Smith 1990; Schlomann et al. 1990; Nikodem et al. 2003). The conversions to CCs are usually followed by *ortho*-cleavage of the aromatic nucleus. The biochemistry of this pathway has been extensively studied, especially for *Pseudomonas knackmussii* sp. B13, a 3-CBA degrading bacterium (Schlomann et al. 1990). In contrast to the modified *ortho* pathway, CCs may be cleaved at the *meta* position by an extradiol dioxygenase. This pathway has been shown to be generally unsuitable for CBA metabolism due to production of suicide metabolites and very few organisms have been reported to productively channel the *meta*-cleavage products into the TCA cycle (Arensdorf an Focht 1995; Mars et al. 1997; Marín et al. 2010).

Alternatively, CBA metabolism may proceed through an initial oxidative or hydrolytic dechlorination reaction (Romanov and Hausinger 1996; Adebusoye and Mileto 2011). This is the most common mechanism for metabolism of 4-CBA and 2,4-CBA. In this reaction, 4-CBA is dechlorinated to 4-hydroxybenzoic acid (4-HBA) which is in turn transformed to protocatechuic acid (PCA) as a central intermediate. Regardless of the degradation mechanism, most CBA degraders use a narrow range of haloaromatics as growth substrates and rarely possess PCB metabolic functionalities. Similarly, studies have also revealed that these organisms only tolerate CBAs at extremely low concentrations and that too at a relatively slow rate of substrate disappearance. Such organisms might not be suitable candidates for cleaning of naturally contaminated systems which often contain high concentrations of these xenobiotics, a factor that may cause significant impairment of microbial degradation (Dua et al. 2002).

In this study, the metabolism of 4-CBA at elevated concentrations by a *Cupriavidus* sp., strain SK-3 was

investigated, and subsequently, an attempt was made to unravel the metabolic pathway leading to mineralization of the acid. Strain SK-3 is an unusual PCB-degrader with the ability to grow on all three monochlorobiphenyls (CBs), 2,4′-, and 2,2′-dichlorobiphenyl (diCB) (Kim and Picardal 2000, 2001). While 2,4′-diCB and 4-CB were completely mineralized, the other PCB congeners utilized were transformed to corresponding CBAs as dead-end products. The metabolic route of degradation of such compounds is still an important consideration when designing bioremediation strategies since the conversion of CBAs into unproductive metabolites may impede PCB metabolism and be toxic to the immediate microbial communities (Vrana et al. 1996; Adebusoye et al. 2008). The findings revealed the existence of at least three major pathways for degradation of aromatic compounds and offer possible reasons why other CBA congeners were not metabolized by strain SK-3.

Materials and methods

Bacteria and culture conditions

Cupriavidus sp. strain SK-3 was isolated from PCB-contaminated, lagoon sludge from a municipal wastewater treatment plant via enrichment with PCB mixtures as a sole source of carbon and energy (Kim and Picardal 2000). The bacterium was routinely grown in minimal salts (MS) medium described by Dorn et al. (1974). The MS medium which was supplemented with 3 mM benzoate (BA), 4-CBA or other selected growth substrates, was further fortified with 1 ml l^{-1} trace element solution without iron salts and EDTA (Pfennig and Lippert 1966). Stock cultures were maintained in 25 % glycerol at −80 °C. If not otherwise indicated, cultivation was carried out aerobically in 100-ml MS medium in 500-ml Erlenmeyer flasks which were shaken on a gyratory shaker incubator (150 rpm) at 27 °C. Cells harvested during late exponential growth phase by centrifugation (10,000×*g*; 10 min at 10 °C) were washed twice in 50 mM potassium phosphate buffer (pH 7.5). Cell pellets were resuspended in the same buffer and stored on ice for oxygen uptake experiments. Cells for growth experiments were washed twice in MS medium and resuspended to a final OD (600 nm) of 2.0.

Substrate diversity and growth dynamics

To ascertain the range of substituted BAs that strain SK-3 used for growth, 20 ml of MS medium in a 100-ml flask was inoculated with a washed cell suspension of the organism pre-grown on 2 mM BA. The following compounds were screened as growth substrates: phenol, 4-chlorophenol, 4-chlorosalicylate, 3-chloro-4-hydroxybenzoate, chloroacetate, 3-HBA, 4-HBA, 2,4-HBA, 4-hydroxybiphenyl, gentisic acid, PCA, salicylate, 4-hydroxybenzoate methyl ester and other potential intermediates of the 4-CBA metabolic pathway. These substrates were supplied at 2 mM concentration. Growth was scored as positive if, after a 7- to 14-day incubation, turbidity was notably greater than controls lacking the test compound, and HPLC analysis showed loss of the target compound.

To follow biodegradation of 4-CBA, the cell suspension was added to 20 ml MS medium supplemented with the acid at concentrations ranging from 1 mM to 10 mM. Samples were withdrawn at suitable time points and analysed for chloride release and via HPLC. Growth was monitored spectrophotometrically at 600 nm and by protein assay. For HPLC analysis, the culture fluid was centrifuged at 10,000×*g* for 10 min, filtered, and collected in glass vials covered with Teflon-lined screw caps.

Oxygen uptake experiments

Conventional Warburg respirometers were used to quantify oxygen consumption. Potassium hydroxide (200 µl) was added to the center well of each flask containing 2 ml of cell suspension. A filter paper strip was included in the center well to soak in the KOH while the side arm contained 200 µl of 3 µmol of the substrate dissolved in 50 mM potassium phosphate buffer (pH 7.5). Oxygen uptake rates were corrected for the endogenous respiration of the cell suspension. All experiments were conducted at 30 °C in one atmosphere with a shaking speed of 110 rpm.

Preparation of cell extracts

Bacterial cells were harvested during the late exponential phase of growth by centrifugation at 800×*g* for 20 min at 4 °C, and washed twice with 100 mM Tris–HCl buffer (pH 8.0) containing 0.1 mM 1,4-dithiothreitol. Pelleted cells were resuspended in the same buffer.

Following the addition of a trace amount of DNase I, the cells were disrupted by two passages through a chilled (4 °C) French pressure cell (Aminco, Silver Spring, MD, USA) at 20,000 lb in^{-2}. Unbroken cells and cell debris were removed by centrifugation at 100,000 × *g* for 1 h at 4 °C. The resulting clear supernatant was stored on ice until use as a source of crude cell-free extracts (CFE) for enzyme assays. The protein contents of the extracts were determined with Coomassie brilliant blue with bovine serum albumin used as the standard.

Enzyme assays

All enzymes were assayed spectrophotometrically at 25 °C in a total reaction volume of 1.0 ml contained in 1-ml quartz cuvettes with a 1-cm light path. In all cases a unit of enzyme was defined as the amount of enzyme necessary to catalyze 1.0 µmol of substrate per min, while specific activities were expressed as µmol of substrate utilized per min per mg of protein under assay conditions.

Catechol-1,2-dioxygenase was assayed according to the protocol described by Dorn et al. (1974) by measuring the presence of *cis, cis*-muconic acid at 260 nm ($\varepsilon = 16{,}800\ M^{-1}\ cm^{-1}$). The assay mixtures contained 1.2 mM EDTA, 33 mM Tris–HCl buffer (pH 8.0) and 0.3 mM catechol or the chloro-substituted analogs. The reaction was initiated by the addition of 20 µl of CFE.

The method of Mars et al. (1997) with slight modifications was used to detect the activity of catechol-2,3-dioxygenase. The activity was determined by measuring the product of catalysis, 2-hydroxymuconate semialdehyde at 375 nm ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$). The reaction mixtures contained 45 mM potassium phosphate buffer (7.5) and 0.3 mM catechol. The reaction was initiated by the addition of 20 µl of CFE which had been heat treated at 55 °C for 10 min.

For the determination of dienelactone hydrolase activity, the protocol of Schlomann et al. (1990) was followed. The method quantified the decrease in substrate concentration at 280 nm ($\varepsilon = 17{,}000\ M^{-1}\ cm^{-1}$) as a result of conversion to maleylacetate. The assay mixtures contained 1.0 mM histidine-HCl (pH 6.5), and 0.1 mM *cis*-dienelactone. The reaction was initiated by the addition of 20 µl of CFE.

The detection of succinyl-CoA-magnesium complex at 305 nm ($\varepsilon = 25{,}000\ M^{-1}\ cm^{-1}$) was used to determine the activity of 3-oxoadipate:succinyl-CoA transferase. The reaction mixtures contained 35 mM Tris–HCl buffer (pH 8.0), 0.7 mM $MgCl_2$, 0.35 mM 3-oxoadipic acid and 0.5 mM succinyl-CoA. The reaction was initiated by the addition of 20 µl of CFE.

The activity of gentisate-1,2-dioxygenase was assayed by monitoring the formation of maleylpyruvic acid from gentisic acid at 340 nm ($\varepsilon = 10,200\ M^{-1}\ cm^{-1}$; Wheelis et al. 1967). The assay mixtures contained 33 mM Tris–HCl buffer (pH 8.0), 1.6 mM $FeSO_4$ and 0.3 mM gentisic acid. The reaction was initiated by the addition of 20 µl of CFE.

PCA-3,4-dioxygenase activity was quantified according to Stanier and Ingram (1954) at 290 nM ($\varepsilon = 2300\ M^{-1}\ cm^{-1}$), where a net reduction in absorbance could be detected due to consumption of PCA and production of 2-carboxy-cis,cis-muconic acid. The reaction mixtures contained 33 mM Tris–HCl (pH 8.0), and 0.3 mM PCA. The reaction was started by the addition of 20 µl of CFE.

In order to determine PCA-4,5-dioxygenase activity, the formation of 2-hydroxy-4-carboxymuconate-6-semialdehyde was monitored at 410 nm ($\varepsilon = 9700\ M^{-1}\ cm^{-1}$; Wheelis et al. 1967). The reaction mixtures contained essentially the same constituents in PCA-3.4-dioxygenase.

Using the same assay mixtures described for PCA-3,4-dioxygenase, the activity of PCA-2,3-dioxygenase was determined by measuring the increase of absorbancy at 350 nm as a result of formation of 2-hydroxy-5-carboxymuconate-6-semialdehyde ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$; Crawford et al. 1979).

To determine 4-HBA-3-monooxygenase activity, the protocol of Marks et al. (1984) was followed by measuring the decrease in absorbance at 340 nm owing to substrate-dependent oxidation of NADH ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$). The assay mixtures contained 40 mM potassium phosphate buffer (pH 7.0), 0.1 mM 4-HBA, 0.08 mM FAD and 0.1 mM NADH. The reaction was initiated by the addition of 20 µl of CFE.

Resting cell assay and metabolite recovery

4-CBA-grown cells were washed twice in 50 mM potassium phosphate buffer (pH 7.5) and resuspended in the same buffer to a final OD ($\lambda_{600\ nm}$) of 2.0 ($\sim$0.45 mg ml^{-1} of protein). Aliquots of 20 ml were dispensed in 100-ml flasks and incubated for 15 min at 15 rpm before 4-CBA was added to give a final concentration of 400 µmol. Incubations without cells and with heat inactivated cells were used as controls.

Metabolism was terminated at specific intervals by H_2SO_4 acidification and subsequent centrifugation to free culture broth of cells. Production of metabolic products was monitored by HPLC.

Analytical techniques

Inorganic chloride released into culture fluids was determined turbidimetrically by measuring AgCl precipitation at 525 nm (Hickey and Focht 1990). Chloride was quantified by reference to a five-point calibration curves that was linear from 0.1 to 3.0 mM. The typical correlation coefficient, r^2, for standard curves was 0.95–0.99.

Residual concentrations of substrates and those of recovered metabolic products in culture fluids were followed by HPLC (Merck-Hitachi chromatograph system) equipped with a programmable UV diode array detector. Separations were performed on a LiChropher SC 100 RP18 reversed-phase column (125 by 4.6 mm, i.d.; 5 µm particle; Knauer GmhH Scientific Equipment, Berlin, Germany) by isocratic elution with a mobile phase constiting of a mixture of 2 g l^{-1} *ortho*-phosphoric acid in 80 % methanol at a flow rate of 0.8 ml per min. Injection volume was 10 µl. The column effluents was monitored at 210–280 nm and identified with authentic standards by retention time.

Chemicals

Halocatechols, *cis*-dienelactone, 4-CBA-CoA and 4-HBA-CoA were all obtained from Chemische Mikrobiologie laboratory, Bergische Universität, Wuppertal while biochemicals were procured from Boehringer Mannheim Biochemicals (Mannheim, Germany). CBA congeners were all purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals including those for MS medium formulation, buffer solutions and solvents were of analytical-grade quality and were purchased from Fluka, Merck and Sigma-Aldrich. These chemicals were used without further purification.

Results

Substrate spectrum of strain SK-3

To study the metabolic versatility of *Cupriavidus* sp. strain Sk-3 more directly, the disappearance of various halobenzoates, and other non-aromatic compounds from culture fluids was evaluated both qualitatively and quantitatively. 4-CBA-grown cells readily catabolize 4-bromobenzoic acid (4-BBA), 4-fluorobenzoic acid (4-FBA), in addition to those chemicals described previously by Kim and Picardal (2000, 2001). Similar results were obtained from cells pre-grown on 4-BBA or 4-FBA. Although activities varied depending on the carbon source used for growth, no significant variation was observed in the relative rates of consumption of the halobenzoic acids. It is noteworthy, however, that 4-FBA appears to enter metabolism more readily compared to the other two isomers. All other congeners of CBA, FBA or BBA were not utilized as carbon sources. Nevertheless, the organism could readily transform 2 mM of 2-, 3-, 2,3-, 2,5-, 3,4- and 3,5-CBA to their respective CCs as indicated by substrate disappearance and HPLC detection of the metabolites. These intermediates were not transformed further. Intense dark brown colouration of the culture fluids was clearly observed following extended incubation due to the accumulation and autooxidation of CCs. The products absorbed strongly at 250–260 nm when the cell-free supernatants were scanned spectrophotometrically, which was consistent with the absorption characteristics of CCs. In addition to CCs accumulation, <10 % of the added CBAs was dehalogenated by the cells. 2,4-CBA was not metabolized even when cultivated in the presence of BA or 4-CBA as a cometabolic substrate and irrespective of the concentration supplied as well as the length of incubation. Similar trends were observed for 2,6-CBA and 3,4-BBA. Whereas sparing growth was observed on chloroacetic acid, no growth or chloride release occurred with 4-chlorosalicylic acid, 3-chloro-4-hydroxybenzoic acid and chlorophenols. Growth on TCA cycle intermediates, such as citric acid, acetic acid, or succinic acid, was very rapid, as was growth on potential products of aromatic metabolism including PCA, 3-HBA, 4-HBA, salicylic acid, catechol, gentisic acid and 4-hydroxybenzoic acid methyl ester. It should be noted that the metabolic capabilities of strain SK-3 was not hampered during repeated cultivation on non-inducible media such as nutrient broth, LB broth and peptone.

Growth dynamics of strain SK-3 on 4-CBA concentrations

The growth characteristics of strain SK-3 was evaluated in varying concentrations of 4-CBA (1–10 mM).

Growth was scored by monitoring the optical density, total viable counts as well as protein yield as a function of total biomass concentration. In the controls with heat inactivated cells, degradation of 4-CBA did not occur nor was chloride excreted into the culture medium. Therefore, losses due to abiotic factors were totally eliminated from the experimental setup. Interestingly, the organism showed maximum growth at a concentration of 5 mM, whereas no growth was observed at 4-CBA concentrations of 6–10 mM. As summarized in Fig. 1 and Table 1, the complete degradation of 1–5 mM 4-CBA occurred within 96–172 h of incubation, with specific growth rates and doubling times strictly dependent upon the concentration of the substrate used. Evidence suggests that the initial reaction involves a dehalogenase catalyzed elimination of chloride from the aromatic backbone. In the course of incubation, 4-HBA was temporarily excreted at levels ranging from 0.1 to 0.2 mM. Generally, no appreciable intermediate products accumulated since 4-HBA disappeared very rapidly. When SK-3 cells were incubated with 1–2 mM 4-CBA, growth was exponential with concomitant release of chloride (Fig. 1, panel a). While 1 mM was utilized in 96 h, it took an additional 24 h to accomplish similar fit with 2 mM. In the latter case, no significant attack of 4-CBA was observed within the first 24 h of growth suggesting that active metabolism occurred between 24 and 120 h of cultivation. Although strain SK-3 metabolized 3-mM of the acid in less than 120 h, the growth characteristics were not too different from those obtained when the concentration was increased to 4 mM. Growth and chloride elimination lagged significantly for nearly 36 h. The organism exhibited a more pronounced lag phase when the concentration was further increased to 5 mM (see Fig. 1d). In all incubations, a linear relationship between 4-CBA metabolized and the amount of protein produced was obtained in spite of the noted effects of 4-CBA concentration on growth rate (Fig. 2). In other words, increase in substrate concentration resulted in geometric increase in protein production. In addition, excretion of organically bound chlorine was stoichiometric and mirrored growth and substrate disappearance.

While it is obvious that a gradual increase in concentrations of 4-CBA resulted in pronounced adaptation and extended period of incubation, it is not surprising that the doubling times observed at 3, 2 and 5 mM concentrations were relatively lower than those determined for 1 and 4 mM (Table 1). Similarly, the specific dechlorination activity at lower concentrations was more than twice those obtained at concentrations of 4 and 5 mM. Comparison of the kinetic data revealed that growth varied significantly at $P < 0.05$ level of confidence. However, no such statistical differences existed for substrate mineralization rates as well as chloride elimination from the aromatic ring.

Oxygen uptake studies

The inducibility of 4-CBA transformation activity of washed cell suspensions of strain SK-3 was evaluated for their oxidative potentials for a number of aromatic compounds including derivatives of CBA and structurally-related compounds, as well as a number of potential metabolic products following growth on 4-CBA, 4-HBA, BA, acetic acid and succinic acid (Table 2). 4-CBA-grown cells readily oxidized this acid, BA, and 4-HBA at relatively equal rates. Whereas oxidation of PCA occurred at approximately half the rate obtained for 4-CBA, gentisic acid, 3-HBA, catechol, succinic acid and acetic acid were slowly oxidized (uptake rates generally less than 30 % of the growth substrate). Growth with BA and 4-HBA produced higher induction rates compared with cells pre-grown on 4-CBA with the exception of PCA. Oxidation of 3-HBA, for example, was more inducible than BA, even when growth was at the expense of the latter. Similarly, the oxygen uptake rate for gentisic acid was higher than PCA. Interestingly, oxidation of 4-CBA in 4-HBA incubations was comparable with cells pre-grown on the CBA, while oxygen consumption rate for PCA was nearly twice as high as those obtained for BA- and 4-CBA-grown cells. In comparison, catechol was poorly oxidized in these incubations. In contrast to cells grown on aromatic substrates, oxidation rates of 3-HBA, 4-HBA, 4-CBA and BA diminished significantly for succinic acid- and acetic acid-grown cells, whereas increased relative activity levels were observed for gentisic acid, catechol and to a lesser extent PCA. This suggests that the synthesis of the first catabolic enzyme was induced only during growth on the aromatic substrates. Other congeners such as 2-, 3-, 2,3-, 2,4- and 3,4-CBA, which were not utilized as carbon sources, were all oxidized, albeit very poorly, by cells pre-grown with 4-CBA, BA, and

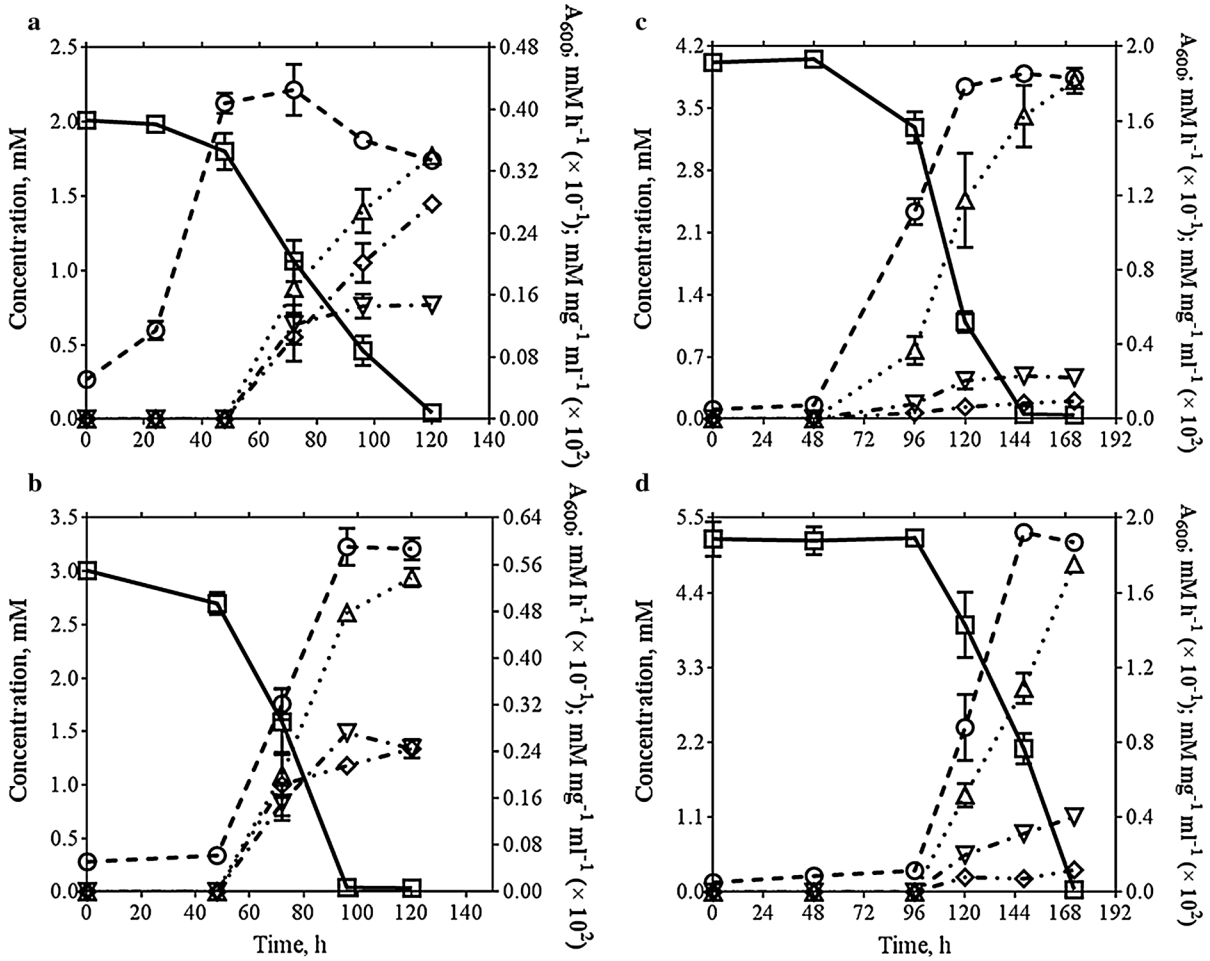

Fig. 1 Kinetics of growth of strain SK-3 in 2 mM, **a**; 3 mM, **b**; 4 mM, **c** and 5 mM, **d** of 4-CBA in batch cultures. Cultures were inoculated with late log-phase cell from batch cultures grown on 3 mM BA. Growth (*circle*) was monitored by optical density at 600 nm, utilization of 4-CBA (*rectangle*) was determined by HPLC, chloride (*triangle*) was quantified spectrophotometrically as described in the text and this parameter was used to determine the rate of mineralization (*inverted triangle*) while specific dechlorination activity (*diamond*) was measured as a function of net chloride concentration per net biomass concentration. Growth of the organisms and chloride elimination (though stoichiometry) lagged significantly with increasing substrate concentration. The data points are averages based on triplicate experiments

4-HBA. In fact, cells grown with the last two carbon sources exhibited relatively higher uptake rates for these compounds. Irrespective of the growth substrates however, strain SK-3 failed to induce the oxidation of 4-chlorophenol, 3-chloro-4-hydroxybanzoic acid, 4-chlorosalicylic acid and all CCs tested.

Enzyme activities in cell-free extracts

To confirm whether degradation of 4-CBA proceeds through (chloro)catechol, PCA or gentisic acid, the presence of key enzymes involved in the *meta*-, *ortho*-, and modified *ortho*-cleavage pathways for the catabolism of these compounds was determined in crude extracts made from cells previously grown on 4-CBA, BA, 4-HBA, acetic acid or succinic acid. Typical results obtained and as presented in Table 3 are representatives of a single experiment. Nevertheless, relative values were consistent between repetitions. With few exceptions, 4-CBA-grown cells exhibited significantly higher specific activities for most key enzymes analysed than extracts from other growth sources. Activities of the enzymes of the *meta*-cleavage pathway were not detectable in all extracts. This was also the case for enzymes of the modified *ortho*-cleavage catabolic route, often involved in the

Table 1 Protein yields of strain SK-3 grown on various concentrations of 4-CBA and chloride eliminated into the culture fluids

4-CBA Concentration (mM)	Incubation period (h)	T_d (h)	Protein yield (mg ml^{-1})	% Substrate metabolized	Cl^{-1} release (mM)	% MZT	ADR (mM h^{-1})	Specific dechlorination activity (mM mg^{-1} ml^{-1})
1.0	96	16.26	0.06 ± 0.0	98 ± 1.04	0.97 ± 0.06	97 ± 5.84	0.007 ± 0.0	27.45 ± 1.29
2.0	120	11.86	0.10 ± 0.01	98 ± 0.52	1.77 ± 0.05	89 ± 2.29	0.007 ± 0.0	27.82 ± 0.48
3.0	120	14.30	0.13 ± 0.01	99 ± 0.07	2.94 ± 0.09	98 ± 2.85	0.013 ± 0.0	24.47 ± 1.57
4.0	172	17.24	0.42 ± 0.01	99 ± 0.02	3.81 ± 0.14	95 ± 3.54	0.012 ± 0.0	9.54 ± 0.15
5.0	172	14.92	0.43 ± 0.0	99 ± 0.06	4.82 ± 0.09	96 ± 1.71	0.015 ± 0.0	11.80 ± 0.13

Mean doubling time was estimated from t = 0–48 h, 48–96 h, 48–120 h for 1–2, 3 and 4–5 mM respectively

MZT mineralization, *ADR* approximate dechlorination rate

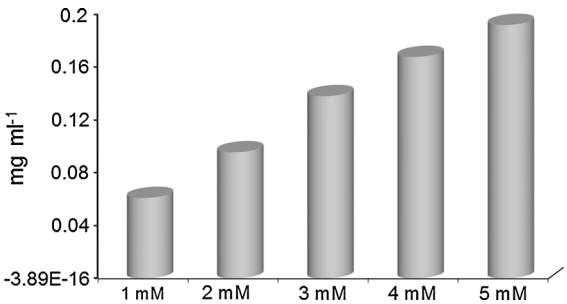

Fig. 2 Protein yield as a function of biomass production during growth of strain SK-3 on various concentrations of 4-CBA. Cultures were inoculated with late log-phase cell from batch cultures grown on 3 mM BA. The data points are averages based on triplicate experiments

catalyses of CCs. Although all extracts demonstrated significant PCA-3,4-dioxygenase activity, gentisic acid-1,2-dioxygenase and catechol-1,2-dioxygenase were also active but induction was generally very poor and respectively ranged insignificantly from 3.3 to 14 % and 0–4.9 % of the levels obtained for PCA-3,4-dioxygenase in 4-CBA-grown cells. These observed enzyme activities indicate that strain SK-3 does not use a *meta*-cleavage pathway to degrade 4-CBA, nor does it utilize the (chloro)catechol or gentisic acid pathway. Further reinforcing this finding was the detection of an NADH-dependent 4-HBA-3-monooxygenase in extracts of cells pre-grown with 4-CBA, BA or 4-HBA, at higher activity with the latter than the former two substrates. This enzyme catalyzes the conversion of 4-HBA to PCA. Consistent with induction of enzymes of the 3-oxoadipate pathway, was the lack of activity against dienelactone, a key product of the modified *ortho*-cleavage route. This is not surprising since growth of strain SK-3 with other substituted BAs resulted in production of CCs as dead-end metabolites. The detection of catechol-1,2-dioxygenase, gentisic acid-1,2-dioxygenase and PCA-3,4-dioxygenase in extracts made from cells grown on succinic acid and acetic acid is an indication of their constitutive expression. However, this expression can be improved when an appropriate enzyme substrate is supplied.

Resting cell assay and metabolite identification

To identify some key metabolites of the catabolic pathway, samples collected from a growing culture of strain SK-3 fed with 4-CBA were screened by HPLC. Samples collected at the onset of the experiment revealed only the presence of the acid as a single chromatographic peak. However, those collected at subsequent sampling points showed the presence of one additional peak displaying similar chromatographic behaviour, i.e., the same retention time and co-elution with an authentic standard of 4-HBA. The concentration of 4-HBA recovered at any given time point never exceeded 200 μM irrespective of the concentration of the initial amount of 4-CBA supplied. No other metabolic products were recovered. By the time the substrate was completely consumed, the level of 4-HBA diminished significantly and was no longer detectable. To detect other possible products of the metabolic pathway, 4-CBA-grown resting cells were used. Transformation of the acid which commenced almost immediately allowed an hourly sampling and analysis of the culture supernatant along with authentic standards of the putative intermediates (Fig. 3).

Table 2 Specific rates of oxygen uptake with halobenzoates and other structurally related compounds by freshly harvested cells of strain SK-3 pre-grown on various carbon sources

Assay substrate	Specific oxygen uptake rates[a] after growth on				
	4-CBA	Benzoate	4-HBA	Acetate	Succinate
Benzoate	98.2 ± 2.93 (77)	133.3 ± 1.52 (100)	146.3 ± 2.07 (105)	31.3 ± 5.66 (38)	60.1 ± 12.15 (59)
2-CBA	9.6 ± 1.76 (8)	27.0 ± 1.43 (20)	68.6 ± 9.99 (49)	1.7 ± 1.28 (2)	7.5 ± 4.52 (7)
3-CBA	13.7 ± 4.74 (11)	40.5 ± 5.0 (30)	71.4 ± 9.37 (51)	8.9 ± 5.03 (11)	5.1 ± 2.99 (5)
4-CBA	127.8 ± 5.26 (100)	101.1 ± 7.70 (76)	120.0 ± 1.96 (86)	20.5 ± 4.96 (25)	11.2 ± 0.62 (11)
2,3-CBA	5.6 ± 4.26 (4)	26.9 ± 9.60 (20)	10.9 ± 5.92 (8)	6.4 ± 2.84 (8)	7.4 ± 2.10 (7)
2,4-CBA	10.5 ± 3.10 (8)	29.6 ± 3.03 (22)	37.7 ± 10.59 (27)	0 (0)	0 (0)
3,4-CBA	22.8 ± 3.05 (18)	46.6 ± 9.0 (35)	19.2 ± 4.94 (14)	4.6 ± 3.21 (6)	6.6 ± 4.41 (6)
4-Chlorophenol	4.9 ± 2.61 (8)	1.6 ± 0.76 (1)	7.7 ± 1.21 (6)	4.3 ± 3.04 (5)	6.7 ± 0.27 (7)
Chloroacetate	10.6 ± 2.30 (8)	33.9 ± 11.68 (25)	37.1 ± 2.39 (27)	5.8 ± 5.14 (7)	4.6 ± 1.05 (5)
Phenol	2.0 ± 0.64 (2)	34.0 ± 5.07 (26)	47.5 ± 6.03 (34)	5.5 ± 2.90 (7)	14.9 ± 7.15 (15)
3-Hydroxybenzoate (3-HBA)	20.4 ± 4.15 (30)	134.6 ± 6.58 (101)	37.1 ± 4.11 (27)	13.1 ± 1.57 (16)	15.5 ± 1.08 (15)
4-Hydroxybenzoate (4-HBA)	92.2 ± 4.06 (72)	123.3 ± 3.89 (93)	139.0 ± 13.36 (100)	20.4 ± 4.15 (25)	39.9 ± 2.79 (39)
Protocatechuate	52.2 ± 4.38 (41)	40.8 ± 3.13 (31)	86.7 ± 5.06 (62)	32.7 ± 8.21 (40)	63.2 ± 4.0 (162
Gentisate	35.7 ± 0.43 (28)	62.3 ± 0.25 (47)	49.3 ± 9.16 (35)	22.6 ± 0.84 (27)	43.2 ± 3.81 (42)
Catechol	22.0 ± 5.0 (17)	87.2 ± 6.19 (65)	39.9 ± 6.16 (29)	116.2 ± 5.95 (141)	83.2 ± 6.09 (81)
Acetate	33.3 ± 3.54 (26)	43.3 ± 6.76 (33)	65.1 ± 1.95 (47)	82.4 ± 5.69 (100)	39.6 ± 5.14 (39)
Succinate	32.6 ± 9.32 (26)	51.5 ± 4.29 (39	65.3 ± 4.34 (47)	60.2 ± 7.31 (73)	102.2 ± 12.61 (100)

[a] Results represent means of at least three independently performed experiments. Rates (corrected for endogenous respiration) are expressed in nanomoles of oxygen per milligram of protein per minute. Values in parentheses are the rates (percent) relative to that for the growth substrates. No activity was observed with the following substrates: 3-chlorocatechol, 4-chlorocatechol, 3-fluorocatechol, 4-fluorocatechol, 3-bromocatechol, 4-chlorosalicylic acid, 3-chloro-4-hydroxybenzoic acid, 2,6-chlorobenzoic acid or trichlorobenzoic acids

Table 3 Specific catabolic enzyme activities in cell extracts of strain SK-3 following growth on different substrates

Enzyme	Sp act (U mg of protein^{-1}) of enzyme in crude extracts of SK3 pregrown on				
	4-CBA	Benzoate	4-HBA	Acetate	Succinate
Catechol 1,2-dioxygenase					
Catechol	0.309 ± 0.003	0.547 ± 0.023	0.01 ± 0.004	0.358 ± 0.02	0
3-CC	0	0.004 ± 0.0	0	0	0
4-CC	0	0	0.002 ± 0.0	0	0
3-FC	0	0.003 ± 0.0	0.001 ± 0.0	0	0
4-FC	0.005 ± 0.0	0	0.002 ± 0.0	0	0
3-FC	0	0	0.002 ± 0.0	0	0
Catechol 2,3-dioxygenase					
Catechol	0	0	0.002 ± 0.0	0	0
3-CC	0	0.006 ± 0.0	0	0	0
4-CC	0	0	0	0	0
4-FC	0	0	0	0	0
Dienelactone hydrolase	0.005 ± 0.0	0.001 ± 0.0	0	0	0
Protocatechuate dioxygenase					
3,4-	11.281 ± 0.13	0.125 ± 0.005	1.069 ± 0.25	0.496 ± 0.011	0.373 ± 0.013
2,3-	0	0	0	0	0
4,5-	0	0	0	0	0
Gentisate 1,2-dioxygenase	1.535 ± 0.10	0.44 ± 0.02	0.664 ± 0.015	0.627 ± 0.022	
3-Oxoadipate:succinyl-CoA transferase	0.124 ± 0.0	0.02 ± 0.0	0.06 ± 0.0	0	0.02 ± 0.0
4-HBS Hydroxybenoate-3-momooxygenase	0.464 ± 0.01	0.042 ± 0.0	0.811 ± 0.011	0.045 ± 0.0	0.069 ± 0.0

Data presented are means of at least two independently performed experiments

CC chlorocatechol, *FC* fluorocatechol, *BC* bromocatechol, *HBA* hydroxybenzoate

Two different transformation products in addition to 4-HBA were recovered. On comparison of UV–VIS spectra and retention times with the data of authentic standards, these products were identified as PCA and β-carboxy-*cis,cis*-muconic acid (CMA). CMA, which failed to accumulate, was recovered in very minute quantity (40 μM) after nearly 18 h of incubation. Its level diminished subsequently. The isomeric 3-HBA was not present, nor were gentisic acid, CCs or any chlorinated products, unlike when cells were grown on 2-, 3- or 3,5-CBA.

The appearance of 4-HBA within 1 h of cultivation coincided with the initial decrease in 4-CBA concentration and increase in chloride concentration. PCA was detected following accumulation of nearly 240 μM of 4-HBA (Fig. 3). Although it is noteworthy that 4-CBA was completely transformed with concomitant stoichiometric release of chloride, only 65 and 39 % were recovered as 4-HBA and PCA, respectively.

Discussion

Recently characterized as *Cupriavidus* sp. based on the 16S rRNA gene sequencing (Vilo et al. 2014), strain SK-3 is a potent degrader of PCBs. According to Kim and Picardal (2000, 2001), it was the second organism reported to grow on all isomers of CBs as well as 2,4′-diCB. While 2-CB and 3-CB were respectively transformed to 2- and 3-CBA as dead-end products, 4-CB and 2,4′-diCB were completely mineralized most likely through 4-CBA since, this is the only 4-CBA utilized as carbon source. In aerobic microorganisms, the limitation in the range of CBA congeners which can be metabolized is often attributable to the narrow substrate specificity of the first enzyme in the metabolic pathway (Reineke and Knackmuss 1978; Miguez et al. 1990). However, this assumption may not be true for strain SK-3. The fact that 2-, 3- and 3,4-CBA were all rapidly transformed to respective CCs readily suggests a narrow spectrum of

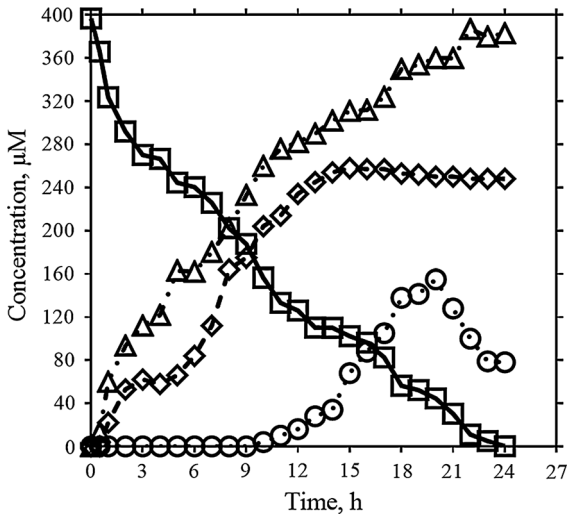

Fig. 3 Rapid transformation and dehalogenation of 4-CBA (*rectangle*) to 4-HBA (*diamond*) and subsequent conversion of the latter to PCA (*circle*) by resting cells of strain SK-3. Chloride released (*triangle*) was stoichiometric and generally mirrored disappearance of the initial substrate. No transformation was recorded in controls inoculated heat inactivated cells or non-inoculated flasks. Concentration of substrate and those of transformed products was analysed by HPLC while chloride was determined turbidimetrically as described in the text. Cells pregrown on 4-CBA were harvested and then resuspended in fresh potassium phosphate buffer ($\sim$0.45 mg ml^{-1} of protein), which was amended with 400 µmol of 4-CBA. The data points are averages based on triplicate experiments

activity of enzymes further downstream of BA-1,2-dioxygenase and that the metabolic bottleneck are either CCs which are unable to serve as substrates for ring fission dioxygenases in the organism or products of the *ortho*-cleavage of these catabolites. Data from respiration experiments indicate that the BA dioxygenase in strain SK-3 also has affinity for a number of substituted analogs. The conclusion was drawn from the fact that all of these halogenated compounds were oxidized, further reinforcing the accumulation of CCs in culture fluids as a bottleneck in degradation. A possible explanation for this inhibitory effect was a proposal that CCs might chelate the iron cofactor for ring-fission dioxygenases which is a necessity for catalysis (Gibson et al. 1968).

Strain SK-3 is no exception to the general rule for 4-CBA metabolism but differs from all other organisms known to metabolize the acid, not only in the fact that it is able to grow on PCBs and all 4-halobenzoic acid isomers but also due to the concentrations of 4-CBA metabolized. Although increasing concentrations of 4-CBA resulted in pronounced adaptation and extended incubation periods (Fig. 1), elimination of chloride from the aromatic nucleus was stoichiometric for all incubations. Therefore, the metabolic capacity of strain SK-3 is superior to that of *Arthrobacter* sp. TM-1 described by Marks et al. (1984) and *Arthrobacter* sp. FG1 (Radice et al. 2007). According to Mark and Co-workers, strain TM-1 was barely able to degrade 2.5 mM of 4-CBA only after exhibiting a 7- h lag time in a reaction that was not rapid. Similar results have been demonstrated for the chlorobenzene-degrading bacterium, strain WR106 (Reineke 1984), for which both the growth rate and the lag time were dependent upon the concentration of chlorobenzene supplied. In a related study, van den Tweel et al. (1986) observed that the growth of *Alcaligenes denitrificans* NTB-1 was a function of 4-CBA concentration and the length of adaptation. But unlike NTB-1, strain SK-3 could metabolize 4-CBA up to a 5 mM concentration without showing any evidence of toxicity and total impairment of metabolic machinery, a clear deviation from other available reports in the literature.

The pathway for 4-CBA degradation by strain SK-3 as depicted in Fig. 4 is proposed to proceed through PCA. Although this is a known route in most organisms, degradation through 4-CC has been reported. The fact that 3-CC or 4-CC was not oxidized and the lack of activity in cell-free-extracts show that the main mechanism for 4-CBA degradation does not involve initial dioxygenation of the aromatic nucleus. Accumulation of CCs in culture fluids of cells incubated with 2-CBA, 3-CBA or 3,5-CBA, ruled out the CC possibility. Therefore, the only reason why SK-3 was able to utilize 4-CBA for growth out of all the congeners tested was the involvement of an initial hydrolytic dehalogenation reaction and subsequent channeling of the products through the PCA pathway and, by so doing, bypassing the CC metabolic route and thereby avoiding production of suicide metabolic products. It would appear that the presence of chlorine at the *ortho* position of the aromatic skeleton is inhibitory to the dehalogenase of SK-3 since 2,4-CBA was neither transformed nor utilized as growth substrate. This is further reinforced by the non-transformation to CCs like other halobenzoic acids.

It would appear that when strain SK-3 is grown on 4-CBA, there is a gratuitous induction of the catechol and gentisic acid pathways even if both are not

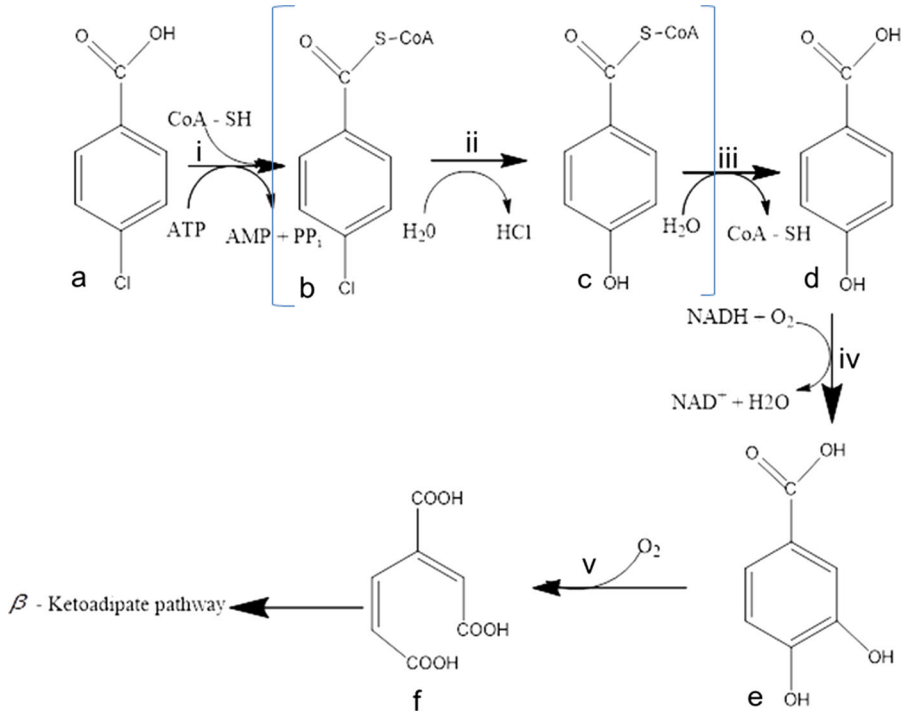

Fig. 4 Proposed pathways for the metabolism of 4-CBA by strain SK-3. 4-CBA, **a**; 4-chlorobenzoyl-CoA, **b**; 4-hydroxybenzoyl-CoA, **c**; 4-HBA, **d**; PCA, **e**; CMA, **f**; by 4-CBA-CoA ligase (*i*), 4-Chlorobanzoyl-CoA dehalogenase (*ii*), 4-hydroxybenzoyl-CoA thioesterase (*iii*), 4-HBA-3-monooxygenase (*iv*), PCA-3,4-dioxygenase (*v*). Metabolites in parenthesis as well as enzymes (*i*), (*ii*) and (*iii*) were not detected in culture fluid but are a probability on the basis of results obtained from other bacteria (Wang et al. 2010) coupled with the assimilation of 4-CBA-CoA and 4-HBA-CoA in resting cell as well as the detection of these genes on the chromosome of the organism. Only activities of enzymes iv and v were assayed from CFE

involved in the metabolism of the substrate. Such simultaneous pathway activation was observed on 3-HBA and 4-HBA during the cultivation of *Alcaligenes* sp. strain L6 on 3-CBA (Krooneman et al. 1996). Grund et al. (1990) observed that, in cells of *Amycolatopsis* sp. incubated with substrates that were metabolized via catechol, enzymes of the PCA pathway were gratuitously induced. Similar phenomenon was also reported for *Rhodococcus erythropolis* during growth on salicylic acid (Suemori et al., 1995). Evidence for simultaneous activations of catechol, PCA and gentisic acid pathways in strain SK-3 is provided by the data obtained from oxygen uptake experiments as well as observed activities of dioxygenases of these substrates. For instance, cell-free extracts of 4-CBA-grown cells exhibited eight-times higher activity of PCA dioxygenase than of gentisic acid dioxygenase (Table 3), whereas, in 4-HBA-grown cells, the two activities were nearly comparable. Moreover, cells pre-grown on BA possess approximate equivalent activity levels of catechol and gentisic acid dioxygenases. Both were nearly three-times higher than PCA dioxygenase. In a parallel experiment it was obvious that both 3-HBA and 4-HBA were respired by 4-CBA-grown cells of SK-3 (though more so in the latter than the former), suggesting that one of these HBAs is an intermediate in the 4-CBA metabolism. However, the metabolites excreted by resting cells implicated 4-HBA and PCA in addition to the *ortho*-cleavage product of the latter which did not accumulate to any appreciable level. All these observations put to rest the route for 4-CBA metabolism and taken together suggest the PCA metabolic pathway. Moreover, the conversion of 4-CBA to 3-HBA may not, be a thermodynamically favourable reaction.

The conversion of 4-CBA through 4-HBA to PCA is not enough justification for completely ruling out the catechol pathway. PCA can serve as substrate for either oxidative decarboxylation to 1,2,4-trihydroxybenzene as previously documented for *Trichosporon cutaneum* (Anderson and Dagley 1980) and *Arxula*

adeninivorans CBS 8244 (Middelhoven et al. 1992) or for non-oxidative decarboxylation to catechol as demonstrated for *A. adeninivorans* LS3 (Sietmann et al. 2010), *Norcacdia* sp. (Dhar et al. 2007) and *Bacillus substilis* (Lupa et al. 2008). Nevertheless, the presence of 4-HBA-3-monoxygenase and PCA-3,4-dioxygenase activities provides a compelling evidence that further metabolism of 4-CBA in strain SK-3 occurs via the PCA-branch of the *ortho*-cleavage pathway. The most critical step in the metabolism of 4-CBA is the transformation of the acid to 4-HBA and expulsion of chloride from the aromatic nucleus. Generally, dehalogenation plays a key role in the biodegradation of many haloaromatic compounds most especially if it is the first step in the catabolic processes. These dehalogenases prepare many chlorinated organics for microbial transformation and mineralization. This type of reaction is catalyzed by hydrolytic dehalogenases replacing the halogen substituent with a hydroxyl group from water as the nucleophile, thus resulting in the formation of a primary alcohol and halide. Although direct evidence from this study is not available, it is possible that 4-CBA is first ligated to CoA by 4-CBA-CoA ligase, which is a substrate for the actual dehalogenation reaction catalyzed by 4-chlorobenzoyl-CoA dehalogenase and subsequent hydrolysis of 4-hydroxybenzoyl-CoA leading to CoA expulsion and formation of 4-HBA by a thioesterase (Wang et al. 2010) in a manner previously reported in *Pseudomonas* sp. CBS3 (Lau and Bruice 2001; Zhang et al. 2001), *Arthrobacter* sp. 4-CBI (Crooks and Copley 1994), and *Arthrobacter* sp. FG1 (Radice et al. 2007). However, the fact that both 4-CBA-CoA and 4-HBA-CoA were metabolized in resting cell assay concomitant with chloride expulsion in near-equimolar quantities in the former (data not shown) coupled with the detection of the genes of the pathway in the chromosome of SK-3 (Vilo et al. 2014) all provide a compelling evidence for the catabolic route constructed in Fig. 4. Interestingly too, Romanov and Hansinger (1996) suggested that the dehalogenases responsible for 4-CBA-CoA dehalogenation are more likely to be widespread in bacterial species. However, the difference in the dehalogenase of strain SK-3 is its extended spectrum of activity and the ability to catalyze 4-FBA, 4-BBA to 4-HBA as well. It should be remembered that cell extracts of strain CBS3 failed to convert 4-FBA (Thiele et al. 1987). This is not surprising since strain SK-3 is able to rapidly utilize both 4-FBA and 4-BBA for growth demonstrating a wider spectrum of activity than those previously reported to dehalogenate 4-halobenzoic acids.

Optimization of bioremediation processes for contaminated systems requires an in-depth knowledge of the pathways involved in degradation of target pollutant most especially, structurally diverse compounds such as CBAs. It will be of critical importance to overcome dead-ends that may be toxic to the degrading organism or other indigenous microflora in the ecosystem. Therefore, it has been demonstrated in this study that the pathway utilized by strain SK-3 in the metabolism of 4-CBA involve an initial hydrolytic dehalogenation and subsequent channeling of the product through PCA which was degraded in the β-ketoadipic acid pathway thus, avoiding production of suicide chlorinated products. The implications of this finding are two-fold: (1) 4-CBA is utilized at a relatively high enough concentration which may impair metabolic functionalities of even capable organisms. This metabolic superiority is associated with the presence of unique and multifunctional dehalogenase with relaxed substrate specificities; and (2) there is gratuitous induction of catechol and gentisic acid pathways when strain SK-3 is grown on 4-CBA, thus suggesting the existence of all major aromatic catabolic routes in a single organism and the use of more than one pathway for degradation of a particular aromatic substrate, i.e., BA. Since contaminated sites usually contain elevated concentrations of pollutants, microorganisms with metabolic capacity for higher concentration of toxic xenobiotics are increasingly suggested as the starting point for development of a successful bioremediation strategy (Thompson et al. 2005; Pandey et al. 2009). Therefore, strain SK-3 could be potentially useful in this regard. In addition, it could be considered a potential source of enzymatic activities that can be exploited to accomplish the degradation or transformation of medium- to highly-substituted haloaromatics which are generally regarded as recalcitrant to microbial degradation.

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Compliance with ethical standards

Conflict of Interest The author declares no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by the author.

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