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Cloning of the *Arthrobacter* sp. FG1 dehalogenase genes and construction of hybrid pathways in *Pseudomonas putida* strains

Francesca Radice · Viviana Orlandi · Valentina Massa · Vanessa Battini · Giovanni Bertoni · Walter Reineke · Paola Barbieri

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Abstract An *Arthrobacter* strain, able to utilize 4-chlorobenzoic acid as the sole carbon and energy source, was isolated and characterized. The first step of the catabolic pathway was found to proceed via a hydrolytic dehalogenation that leads to the formation of 4-hydroxybenzoic acid. The dehalogenase encoding genes (*fcb*) were sequenced and found highly homologous to and organized as those of other 4-chlorobenzoic acid degrading *Arthrobacter* strains. The *fcb* genes were cloned and successfully expressed in the heterologous host *Pseudomonas putida* PaW340 and *P. putida* KT2442 upper TOL, which acquired the ability to grow on 4-chlorobenzoic acid and 4-chlorotoluene, respectively. The cloned dehalogenase displayed a high specificity for *para*-substituted haloaromatics with affinity Cl>Br>I»F, in the order.

Keywords Dehalogenase · *fcb* genes · 4-Chlorobenzoic acid · 4-Chlrotoluene

F. Radice · V. Orlandi · V. Massa · V. Battini · P. Barbieri (✉)
Dipartimento di Biologia Strutturale e Funzionale,
via Dunant 3,
21100 Varese, Italy
e-mail: paola.barbieri@uninsubria.it

G. Bertoni
Dipartimento di Scienze Biomolecolari e Biotecnologie,
Università degli Studi di Milano,
via Celoria 26,
20133 Milan, Italy

W. Reineke
Chemische Mikrobiologie,
Bergische Universität Wuppertal,
Fachbereich C, Gaußstrasse 20,
42097 Wuppertal, Germany

Introduction

Chlorinated aromatic compounds have been introduced into the environment as pesticides, herbicides, and industrial agents and are classified among the most persistent and toxic pollutants, their recalcitrance increasing with the number and the respective position of chlorine substituents on the aromatic ring(s) (Chaudhry and Chapalamadugu 1991). The carbon–chlorine bond is particularly resistant to the cleavage because of the electronegativity of the substituent; therefore, dechlorination is the most important step in chloroaromatic biodegradation (Fetzner and Lingens 1994). Among chloroaromatics, chlorobenzoic acids are known to be intermediates in the microbial breakdown of polychlorinated biphenyls (PCBs), 1,1,1,-trichloro-2,2,-bis(*p*-chlorophenyl) ethane (Häggblom 1992), and various herbicides. Most aerobic bacteria that degrade lower chlorinated PCBs accumulate the corresponding chlorobenzoic acids, especially those with substituents in *ortho* or *para* position, whose degradation could thus represent the rate-limiting step in the mineralization process (Adriaens et al. 1989).

Because of its water solubility and low toxicity, 4-chlorobenzoate (4-CBA) is commonly used as model compound to study haloaromatic biodegradation. A number of bacterial strains, belonging to different genera such as *Arthrobacter*, *Pseudomonas*, *Alcaligenes*, and *Nocardia* (Marks et al. 1984; Thiele et al. 1987; van den Tweel et al. 1987; Klages and Lingens 1979), have been isolated for their ability to grow on 4-CBA as the sole carbon and energy source. A microbial consortium was also described for the dehalogenation of 4-CBA under anoxic conditions (Horowitz et al. 1983).

In aerobic environments, 4-CBA is metabolized through two different pathways: In the first, 4-chlorocatechol is

formed and the chlorine atom removed after the aromatic ring cleavage; in the other, the chlorine is removed directly from the aromatic nucleus, and 4-hydroxybenzoic acid (4-HBA) is formed (Chaudhry and Chapalamadugu 1991; Häggblom 1992). The latter reaction is known as hydrolytic dehalogenation and represents the initial step of 4-CBA degradation in some *Arthrobacter* and *Pseudomonas* strains. Hydrolytic dehalogenation is a sequential reaction catalyzed by 4-CBA–CoA ligase (FcbA), 4-CBA–CoA dehalogenase (FcbB), and 4-HBA–CoA thioesterase (FcbC) (Chang et al. 1992; Zhuang et al. 2003). In *Arthrobacter*, the Fcb encoding genes (*fcb*A, *fcb*B, *fcb*C, in the order) are clustered in an operon, located either on the chromosome or on a plasmid, which is often duplicated. In the 4-CBA degrading *Pseudomonas* sp. DJ-12 and CBS3, the *fcb* genes display a different order (*fcb*BAC) and have been found on the chromosome (Fig. 1; Chae et al. 2000). *Arthrobacter* and *Pseudomonas fcb* genes also are scarcely homologous. The ligase (*fcbA*) and dehalogenase (*fcbB*) encoding genes of the two genera are in fact only 38 and 50% identical, respectively, whereas the thioesterase genes (*fbcC*) appear not to be related (Thoden et al. 2003).

In this work, we report on the isolation and characterization of a new 4-CBA degrading *Arthrobacter* strain and on the construction of recombinant *Pseudomonas* strains able to degrade 4-CBA and 4-chlorotoluene (4-CT).

Materials and methods

Bacteria, media, and growth conditions

Arthrobacter sp. FG1 was isolated from the soil of a contaminated industrial plant by enrichment procedure in mineral medium K1 (Zaitsev and Karasevich 1985) supplemented with 4-CBA as the sole carbon and energy source; after isolation, the strain was maintained on K1 agar plates with 3 mM 4-CBA; long-term maintenance was performed in 25% glycerol at −80°C. *Escherichia coli* JM109 (Yanish-Perron et al. 1985) was currently grown at 37°C in Luria-Bertani (LB) medium (Miller 1972). *Pseudomonas putida* PaW340 (Trp^-, Str^R 4-HBA^+, 4-CBA^-; Franklin et al. 1980) and *P. putida* KT2442 upper TOL (Panke et al. 1998) were routinely grown in LB medium; for growth in mineral medium, *P. putida* PaW340 cultures were supplemented with streptomycin (50 μg/ml) and tryptophan to meet the strain auxotrophy (50 μl/ml). 4-CT was supplied in the vapor phase. If not otherwise indicated, all the bacterial strains were grown at 30°C with shaking. Growth was monitored spectrophotometrically (optical density at 540 nm [$OD_{540\ nm}$]). Cell-free supernatants for chemical analyses were stored at −20°C.

Molecular biology techniques

High molecular weight, total genomic DNA of *Arthrobacter* sp. FG1 was prepared as described by Hopwood et al. (1985). Plasmid DNAs from both *E. coli* and *P. putida* cells were purified by using Qiaprep Spin Plasmid Kit (Qiagen).

Southern hybridization on the total genomic DNA of *Arthrobacter* sp. FG1 restricted with several enzymes was performed using the fluorescent DIG-DNA labeling and detection kit (Roche). The probe was obtained by *Sac*I digestion of the plasmid pAS5 of *Arthrobacter* sp. SU (Schmitz et al. 1992). Digestion with restriction endonucleases and agarose gel electrophoresis were performed by standard methods (Sambrook et al. 1989).

For the amplification of the 16S rRNA gene, the primers 16Sf and 16Sr, which target, respectively, the positions 27

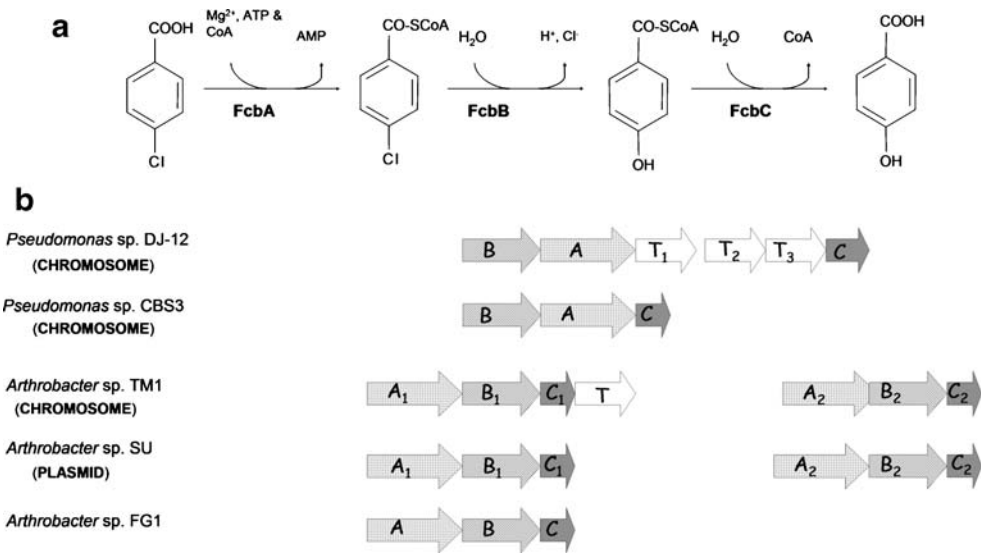

Fig. 1 Hydrolytic dechlorination of 4-CBA (**a**) and arrangement of the *fcb* gene clusters in different *Arthrobacter* and *Pseudomonas* strains (**b**). *fcb*A, *fcb*B, and *fcb*C codes for 4-CBA–CoA ligase, 4-CBA–CoA dehalogenase, and 4-HBA–CoA thioesterase, respectively. Homologous of *fcb*T genes, postulated to encode periplasmic membrane proteins for transportation of 4-CBA (Chae et al. 2000), have not been detected in the DNA fragment cloned from *Arthrobacter* sp. FG1

and 1495 of *E. coli* 16S rRNA gene (Widmer et al. 1998), were used.

Perfectly matching primers MF1 (5′-TCT CGA ACA CGT CGC CCA GGA ACT-3′) and MF3 (5′-CGA CCC TGT CCT AAC AGA CAT CCG-3′) were designed on the basis of the alignment of nucleotide sequences of the *fcb* genes of *Arthrobacter* sp. SU (accession number AF030397) and *Arthrobacter* sp. TM1 (accession number AF042490), obtained using the Clustal W program (Higgins et al. 1994). The polymerase chain reaction (PCR) mix contained 0.4 μg of total genomic DNA of *Arthrobacter* sp. FG1, 0.4 μM of each primer, 0.5 μM of deoxyribonucleoside triphosphates, and 2 U of Triple Master (Eppendorf) with the Tuning buffer supplied. PCR was performed as follows: initial step at 93°C for 3 min, 30 cycles of denaturation at 93°C for 15 s, annealing at 63°C for 30 s, and extension at 68°C for 3 min, final step of extension at 68°C for 10 min.

Nucleotide sequences were determined by using an automatic ABI PRISM 3100 DNA sequencer and deposited in GenBank under the accession numbers AM113544 (16S rDNA) and AM231748 (*fcb* genes).

Plasmid pGEM-t Easy Vector (Promega) was used to clone the amplification product that was then transferred as an *Eco*RI fragment in pUC18 (Vieiria and Messing 1982) and in the RK2-derived pJB3KmD (Blatny et al. 1997) used as cloning vectors for *E. coli* and *P. putida*, respectively. The pUC18 derivative was named pDH4 and the pJB3KmD derivative pDH5. *E. coli* and *P. putida* cells were transformed by electroporation (Sambrook et al. 1989), and recombinants were selected on LB agar plates supplemented, respectively, with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG), 40 μg/ml X-Gal and 100 μg/ml ampicillin or with 50 μg/ml kanamycin.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on crude extracts of *E. coli*/pDH4. Cells were grown at 30°C in LB medium until OD_{540}=0.4, then 0.5 mM IPTG was added and samples withdrawn after 3 h of incubation. Extraction of soluble protein was performed by conventional method (Deutscher 1990). Samples were loaded on 12% acrylamide gel, run for 2 h and Coomassie Blue stained.

Analytical methods

High-performance liquid chromatography (HPLC) analyses of metabolites were performed with a Waters 600E delivery system equipped with a Waters 486 detector and 746 integrator or with a Dionex HPLC (P580 pump, ASI-100 Automated Sample Injector UV/VIS Detector UVD 340S). Reverse-phase chromatography was conducted with a Waters μBondapak C18 column eluted with 40 to 80% methanol/*ortho*-phosphoric acid 2 g/l at a flow rate of 1 ml/min. The compounds were detected by monitoring the A_{254}. Metabolites were identified by comparison of their retention times with those of commercially available standards.

For gas chromatography–mass spectrometry (GC–MS) analysis, the cultural broth was acidified to pH 2.5 and extracted with ethyl acetate. The organic phase was dried with sodium sulfate and then evaporated under vacuum. The sample and the authentic standard were derivatized with *N*-tertbutyldimethylsilyl-*N*-methyltrifluoroacetamide. Mass spectra were recorded by using a Hewlett-Packard 5890 instrument equipped with a MSD-HP 5971A mass spectrometer (column Supelco SPB-5, 30 m, I.D. 0.25 mm, 0.25 μm thickness).

The concentration of chloride ions released from 4-CBA was determined according to Freier (1974). Concentrated HNO_3 (100 μl) and 100 μl of $AgNO_3$ (100 mM) were combined with 800 μl of cell-free samples. After 10 min of incubation, the formation of AgCl was monitored by measuring the absorbance at 546 nm. The chloride concentrations were calculated using a calibration curve.

Enzyme assays

The qualitative colorimetric assay to detect dehalogenase activity is based on the use of pH indicator phenol red dye (Holloway et al. 1998). Bacterial colonies were grown overnight on 0.45-μm pore size nitrocellulose filters (Millipore) on LB agar plates, and then, the filters were transferred on K1 agar plates supplemented with phenol red (13 μg/ml), 4-CBA (3 mM), and IPTG (0.5 mM). After incubation, positive clones were surrounded by a yellow halo and turned yellow themselves because of the acidification caused by HCl formation.

The rates at which *Arthrobacter* and *P. putida* cells dehalogenated 4-CBA were determined by monitoring the change in chloride ion concentration in the medium. An exponentially growing culture was washed twice in 0.1-M phosphate buffer (pH 7.1) and resuspended in the same buffer to obtain an $OD_{540\ nm}$=2. 4-CBA (final concentration 3 mM) was added, and samples were collected at 10-min intervals. Cells were removed by centrifugation, and the chloride ion concentration was determined as described above. Specific activity was expressed as nmol chloride min^{-1} mg^{-1} of protein. Cell protein concentrations were determined by bicinchoninic acid kit for protein determination (Sigma) using bovine serum albumin as the protein standard. Cells were resuspended in 0.1 M NaOH and incubated in boiling water for 20 min before protein concentration assays.

To determine the dehalogenase substrate specificity of *P. putida* PaW340/pDH5, suspensions were prepared as described above, and substrates were added at a final concentration of 3 mM. At 10-min intervals, the supernatants were HPLC analyzed to monitor the substrate decrease.

Results

Microorganism isolation and characterization

A Gram-positive, motile, irregular, often V-shaped rod was isolated from a sample of a contaminated soil from an industrial plant by the enrichment technique in the presence of 4-chlorobenzoic acid (4-CBA) as the sole carbon and energy source. The colonies were circular, entire, convex, and smooth after 48 h of incubation on LB agar at 30°C. Catalase reaction was positive. Spores were not produced, and starch was not hydrolyzed. The 16S rDNA sequence (accession number AM113544) was 97 to 98% identical to that of several *Arthrobacter* type strains, not allowing to assign the new isolate to a given species. The strain was thus designated *Arthrobacter* sp. FG1.

Arthrobacter sp. FG1 was unable to grow on the other chlorobenzoate isomers as well as on chlorinated phenols and toluenes. Among non-chlorinated compounds, it was able to use only a narrow range of substrates, all of them partially oxidized. Growth was observed on 4-hydroxybenzoate, benzoate, benzaldehyde, phenol, and *p*-cresol, but not on toluene, xylenes, and methyl-substituted benzoates.

Cells of *Arthrobacter* sp. FG1 inoculated from LB liquid medium into 3 mM 4-CBA-mineral salts medium grew after a lag period of 25 h, higher concentrations of substrate, up to 6 mM, determined a longer lag phase (data not shown). During the exponential phase, the doubling time was 7.4 h, and the growth was accompanied by 4-CBA disappearance and a concomitant, equimolar chloride release. To identify metabolites of the catabolic pathway, samples were collected from an exponentially growing culture and analysed by HPLC. A compound, showing the same retention time of and coeluting with an authentic standard of 4-HBA, was detected. GC–MS analyses of the metabolite extracted from the culture supernatant confirmed the identification of 4-HBA. The increase in 4-HBA concentration in the cultural broth coincided with the decrease in 4-CBA concentration (not shown) but never exceeded 0.1 mM. When 4-CBA was totally consumed by the cells, no 4-HBA remained. These data suggested that the first step of 4-CBA degradation in *Arthrobacter* sp. FG1 consisted of a hydrolytic dehalogenation.

Analysis of dehalogenase genes and expression in *E. coli*

The presence of sequences homologous to dehalogenase encoding genes in the *Arthrobacter* sp. FG1 genome was demonstrated by Southern blot, using the *fcb* gene cluster of *Arthrobacter* sp. SU (Schmitz et al. 1992) as the probe (not shown).

PCR amplification with the primers MF1 and MF3 resulted in a 4.7-kb product that was cloned in pGEM-t Easy Vector and then transferred as an *Eco*RI fragment to pUC18, giving rise to plasmid pDH4. The 4.7-kb fragment cloned in pDH4 was sequenced; the complete nucleotide sequence (4781 nt) was deposited in GenBank database under the accession number AM231748. Sequence analysis revealed four large open reading frames (ORFs; ORF IV, ORF ABC) transcribed in the same direction. Comparison of the nucleotide and predicted amino acid sequences of ORF A (nt 2411–3427), ORF B (nt 3424–4254) and ORF C (nt 4254–4709) revealed a high degree of identity (99–100%) with the *fcbA*, *fcbB*, and *fbcC* genes and gene products (4-CBA–CoA ligase, 4-CBA–CoA dehalogenase, and 4-HBA–CoA thioesterase, respectively) of other 4-CBA degrader *Arthrobacter* strains retrieved from GenBank, and were thus named *fcb*A, *fcb*B, and *fcb*C, respectively (Fig. 1b).

ORF A is preceded by a putative Shine Dalgarno (AGAA) and a promoter-like sequence (−35 and −10 region). Upstream of ORF A, an additional ORF (ORF IV, [nt 316–1005]), showed 99% identity with ORFs of both *Arthrobacter* sp. SU and *Arthrobacter* sp. TM1, also located upstream of *fcbA* and whose functions are unknown. No homologous of the hypothetical transporter *fcbT* gene was found in the cloned fragment.

SDS-PAGE (not shown) of crude extracts of IPTG-induced *E. coli* JM109/pDH4 cultures allowed to detect two soluble proteins, absent in non-induced samples, whose sizes were consistent with those of FcbA (51 kDa) and FcbB (29 kDa). The expression of dehalogenase activity in *E. coli* JM109/pDH4 was confirmed by the qualitative colorimetric assay described in "Materials and Methods." No dehalogenase activity was detected in *E. coli* JM109 carrying only the cloning vector pUC18.

Cloning and expression of the *Arthrobacter* sp. FG1 dehalogenase genes in *P. putida* strains

Plasmid pDH5 was constructed by transferring the 4.7-kb *Eco*RI fragment cloned in pDH4 to the broad host range vector pJB3KmD. After transformation into *P. putida* PaW340, recombinant clones were selected and subjected to restriction analysis to choose those carrying the *fbc* genes cloned in the same orientation of the pJB3KmD P_{lac} promoter. To verify whether the introduction of the *fcb* genes in *P. putida* PaW340 resulted in a dehalogenase activity, several colonies were subjected to the qualitative assay, but no activity was detectable, possibly because of the low copy number of the vector pJB3KmB used for cloning. However, recombinant *P. putida* PaW340/pDH5 clones grew on 4-CBA supplied as the sole carbon and energy source with a doubling time of 3.5 h. The increase in turbidity was accompanied by 4-CBA disappearance and the concomitant, stoichiometric chloride release.

Plasmid pDH5 was then transformed into *P. putida* KT2442 upper TOL (chromosomal insertion of the *upper* TOL genes [*xylR*/*xylUWCMABN*] from pWW0; Panke et al. 1998), a strain able to oxidize 4-CT to 4-CBA through the progressive oxidation of the methyl group. Recombinant clones were selected on K1 agar plates supplemented with 4-CT in the vapor phase after approx. 15 days of incubation. To confirm the ability of the recombinant clones to utilize 4-CT as the sole carbon and energy source, the growth in mineral liquid medium was measured. To avoid alterations in the substrate concentration when samples were withdrawn from the culture, several independent cultures with increasing concentrations of 4-CT were set up, and after three days of incubation, the turbidity and the chloride concentration of each culture were measured. Both the absorbance of the culture and concentration of chloride resulted proportional to the substrate concentration (Fig. 2), indicating that the growth occurred at the expense of 4-CT.

To better characterize the growth of the recombinant strain, *P. putida* KT2442 upper TOL/pDH5 was inoculated in mineral medium supplied with 4-CT 3 mM in an Erlenmayer flask. Samples were withdrawn from a side Teflon tube and a doubling time of approx. 10 h was estimated. HPLC analyses of samples withdrawn during the growth revealed only transient trace amounts of 4-CBA, whereas no 4-HBA was detectable.

Specific activity and substrate range of the cloned dehalogenase

The specific activity of the *Arthrobacter* sp. FG1 dehalogenase was measured in both *Arthrobacter* sp. FG1 and *P. putida* PaW340/pDH5 resting cells after growth on 4-CBA and was found to be 48.8±1.08 and 109.3±21.4 nmol chloride min^{-1} mg^{-1} of protein, respectively. Dehalogenase activity was not detectable in malate-grown *Arthrobacter* sp. FG1 cells.

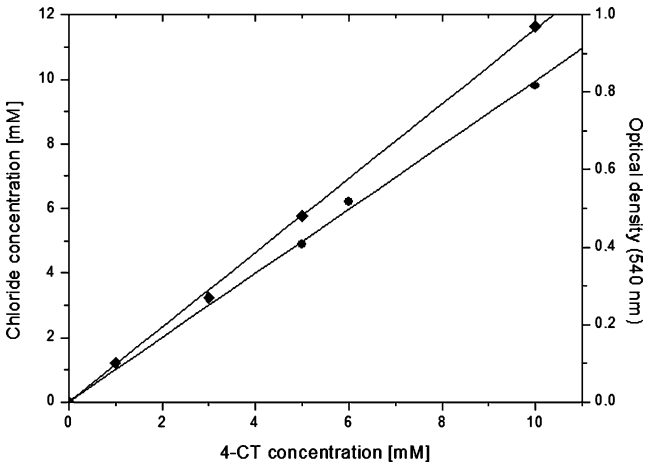

Fig. 2 Increase in turbidity (*diamond*) and chloride release (*circle*) vs 4-CT consumption in batch cultures of *P. putida* KT2442 upper TOL/pDH5. Liquid 4-CT was added to a side arm in small portions over a period of 3 days. The concentration of the substrate was calculated as if it was totally absorbed by the medium

Table 1 Relative activity of hydrolitic dehalogenation of various haloaromatic compounds by *P. putida* PaW340/pDH5 cells grown on 4-CBA

Substrate	Relative activity (%)[a]
4-Chlorobenzoate	100
4-Bromobenzoate	85
4-Iodobenzoate	66
4-Fluorobenzoate	0
4-Methylbenzoate	0
Benzoate	0
3-Chlorobenzoate	0
2-Chlorobenzoate	0
3,4-Dichlorobenzoate	0

[a] Values are expressed as percentage of the activity measured with 4-chlorobenzoate taken as 100%.

The range of substrates of the cloned dehalogenase activity was investigated in the recombinant strain *P. putida* PaW340/pDH5 by monitoring the disappearance of chloro- and other halo-substituted benzoic acids through HPLC analysis. The relative activities toward the tested compounds are expressed as percentage of that determined with 4-CBA and are shown in Table 1. No activity toward halogenated compounds other than 4-CBA, 4-bromobenzoate, and 4-iodobenzoate was detected. Thus, the cloned dehalogenase resulted highly specific for the *para*-substituted haloaromatics and showed a decreasing affinity for substrates substitutes with Cl, Br, I, and F, in the order.

Discussion

Microorganisms able to mineralize simple haloaromatic compounds are considered a potential source of enzymatic activities that can be exploited to achieve the degradation of highly substituted haloaromatics, which are currently considered recalcitrant.

By means of enrichment cultures, we isolated an organism able to utilize 4-CBA as the sole carbon and energy source, which was identified as *Arthrobacter* sp. and designated FG1. In several *Arthrobacter* strains, the first reaction of 4-CBA degradation pathway is a hydrolytic dehalogenation, catalyzed by the *fcb*ABC encoded dehalogenase complex, that leads to the formation of 4-HBA (Marks et al. 1984; Müller et al. 1988; Tsoi et al. 1991; Schmitz et al. 1992; Zhuang et al. 2003). In the 4-CBA degrader *Arthrobacter* sp. FG1, the stoichiometric correlation among 4-CBA disappearance, chloride release, and 4-HBA appearance suggested that the same catabolic route was followed. This hypothesis was further supported by the

isolation of *fcb* genes highly homologous to those found in other *Arthrobacter* strains (Marks et al. 1984; Schmitz et al. 1992). The dehalogenase activity appeared to be inducible and displayed a range of substrates almost identical to that of other known dehalogenases (Müller et al. 1988).

In *Arthrobacter* strains SU and TM1, as well as in *Arthrobacter* sp. FG1, upstream of an ORF (ORF IV) of unknown function, sequences, identified as a partial *tnpA* gene, and an inverted repeat related to those of the IS30 family, were found, which may represent a likely vestigial IS element. In strain TM1, *fcb* genes are located on the chromosome and associated to IS*1409*, which may account for horizontal transfer of *fcb* genes (Gartemann and Eichenlaub 2001). In *Arthrobacter* sp. SU, *fcb* genes are encoded by plasmid pSU (Schmitz et al. 1992), which lacks the IS*1409*; however, a copy of the IS was detected on the SU chromosome (Gartemann and Eichenlaub 2001). Although we neither investigated the genetic location of *fcb* genes nor the presence of IS*1409* in *Arthrobacter* sp. FG1, the extremely high degree of homology among both *fcb* genes and upstream sequences in independent isolates from distinct geographical locations suggests that dehalogenase encoding genes derived from a recent common ancestor and underwent a wide horizontal diffusion in members of this genus.

Cloning of *fcb* genes in *E. coli* already demonstrated that the *Arthrobacter* dehalogenase can be successfully expressed in Gram-negative bacteria (Tsoi et al. 1991). In this paper, cloning of the *Arthrobacter* sp. FG1 *fcb* genes in *P. putida* PaW340, a strain able to grow on 4-HBA, resulted in a recombinant strain able to use 4-CBA as the sole carbon and energy source. In *P. putida* PaW340/pDH5, the dehalogenase specific activity was approximately twofold higher than that measured in *Arthrobacter* sp. FG1 cells.

Cloning of selected enzymatic activities in aromatic compounds degraders has been widely used to obtain bacteria with improved catabolic abilities (Hrywna et al. 1999; Pieper and Reineke 2000). The results described above prompted us to design a new hybrid pathway and to construct the appropriate recombinant strain for 4-CT degradation, a compound that only few microorganisms are able to mineralize. Degradation of 4-CT has been described in wild-type strains as *Ralstonia* sp. PS12 (Pollmann et al. 2001) as well as in mutant or transconjugant *Pseudomonas* strains (Haigler and Spain 1989; Brinkmann and Reineke 1992). In these strains, chlorocatechols, produced by broad-substrate-range peripheral mono- or dioxygenases, are channeled into the so-called modified *ortho* pathway. The modified *ortho* pathway is in fact endowed with enzymatic activities able to accept and productively convert chlorinated intermediates (Reineke 1998). By cloning the *Arthrobacter* sp. FG1 *fcb* genes into *P. putida* KT2442 harboring only the upper part of the TOL pathway, we obtained a strain able to use 4-CT as the sole carbon and energy source through the novel catabolic route depicted in Fig. 3. Because of the absence of the *meta*-cleavage pathway, which includes also the toluate dioxygenase as well as the following dehydrogenase coded on the wild-type TOL plasmid, the formation of a chlorocatechol will not occur. Growth of the recombinant strains on 4-CT will take place, as XylR, the

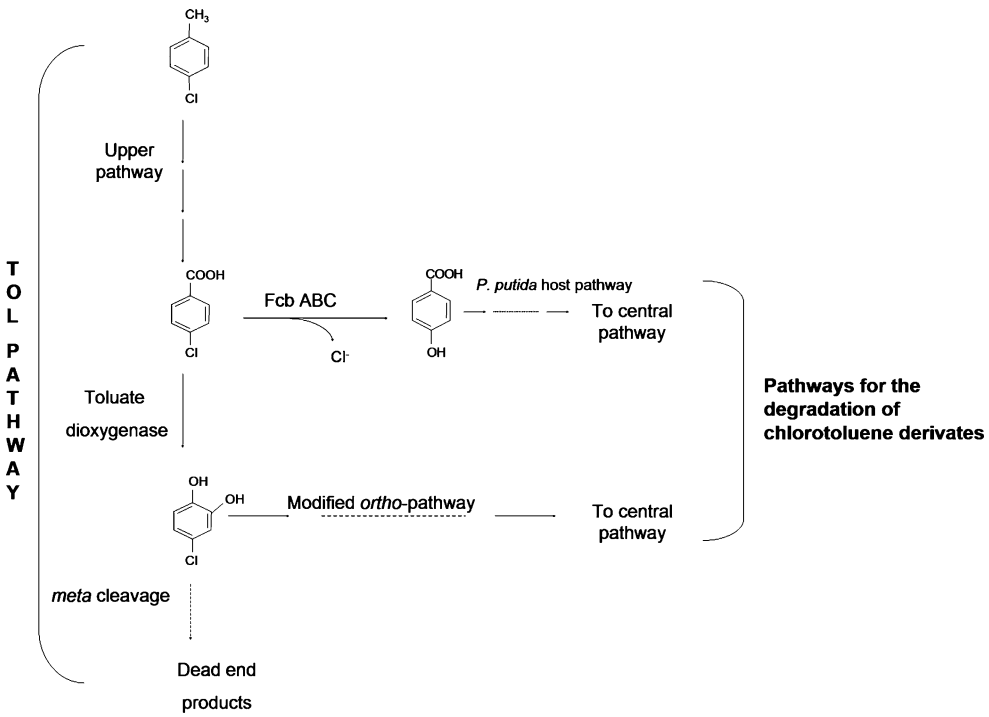

Fig. 3 Hybrid routes for 4-chlorotoluene degradation based on the TOL pathway. The pathway developed by combining the TOL upper pathway with the dehalogenase activity cloned from *Arthrobacter* (*upper section*) is compared with that proceeding through the formation and channeling of 4-chlorocatechol into a modified *ortho* pathway (*lower section*; Reineke and Knackmuss 1980). Misrouting of chlorocathecols into a meta-cleavage pathway can lead to dead-end products and cause cell lethality

regulator of the TOL upper operon, recognizes 4-CT as an effector (Abril et al. 1989).

The expression of *Arthrobacter fcb* genes in a *P. putida* host that gives rise to a degradation pathway for chloroaromatics proceeding through an early dechlorination of the substrate is shown. This result underlines the potential of hydrolytic dehalogenation. The possibility to remove the chlorine atom in an early stage of the catabolic route, thus bypassing the formation of a chlorocatechol whose degradation requires specialized enzymatic sets (Reineke 1998; Kaschabek et al. 1998; Göbel et al. 2004; Nikodem et al. 2003), could be exploited for the rational design of new pathways for chloroaromatic compound degradation.

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