Degradation of chloroaromatics by Pseudomonas putida GJ31

Kunze, Markus; Zerlin, Kay F.; Retzlaff, Alexander; Pohl, Jens O.; Schmidt, Eberhard; Janssen, Dick; Vilchez-Vargas, Ramiro; Pieper, Dietmar H.; Reineke, Walter

Published in:
Microbiology-Sgm

DOI:
10.1099/mic.0.032110-0

Publication date:
2009

Microbiology (2009), **155**, 4069–4083 **DOI** 10.1099/mic.0.032110-0

Degradation of chloroaromatics by *Pseudomonas putida* GJ31: assembled route for chlorobenzene degradation encoded by clusters on plasmid pKW1 and the chromosome

Markus Kunze,[1]† Kay F. Zerlin,[1]‡ Alexander Retzlaff,[1] Jens O. Pohl,[1] Eberhard Schmidt,[1] Dick B. Janssen,[2] Ramiro Vilchez-Vargas,[3] Dietmar H. Pieper[3] and Walter Reineke[1]

Correspondence
Walter Reineke
reineke@uni-wuppertal.de

[1]Bergische Universität Wuppertal, Chemical Microbiology, D-42097 Wuppertal, Germany

[2]Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, 9747 AG Groningen, The Netherlands

[3]Department of Microbial Pathogenesis, Helmholtz Centre for Infection Research, D-38124 Braunschweig, Germany

Received 25 June 2009
Revised 4 September 2009
Accepted 8 September 2009

Pseudomonas putida GJ31 has been reported to grow on chlorobenzene using a *meta*-cleavage pathway with chlorocatechol 2,3-dioxygenase (CbzE) as a key enzyme. The CbzE-encoding gene was found to be localized on the 180 kb plasmid pKW1 in a *cbzTEXGS* cluster, which is flanked by transposases and encodes only a partial (chloro)catechol *meta*-cleavage pathway comprising ferredoxin reductase, chlorocatechol 2,3-dioxygenase, an unknown protein, 2-hydroxymuconic semialdehyde dehydrogenase and glutathione *S*-transferase. Downstream of *cbzTEXGS* are located *cbzJ*, encoding a novel type of 2-hydroxypent-2,4-dienoate hydratase, and a transposon region highly similar to Tn*5501*. Upstream of *cbzTEXGS*, *traNEOFG* transfer genes were found. The search for gene clusters possibly completing the (chloro)catechol metabolic pathway of GJ31 revealed the presence of two additional catabolic gene clusters on pKW1. The *mhpRBCDFETP* cluster encodes enzymes for the dissimilation of 2,3-dihydroxyphenylpropionate in a novel arrangement characterized by the absence of a gene encoding 3-(3-hydroxyphenyl)propionate monooxygenase and the presence of a GntR-type regulator, whereas the *nahINLOMKJ* cluster encodes part of the naphthalene metabolic pathway. Transcription studies supported their possible involvement in chlorobenzene degradation. The upper pathway cluster, comprising genes encoding a chlorobenzene dioxygenase and a chlorobenzene dihydrodiol dehydrogenase, was localized on the chromosome. A high level of transcription in response to chlorobenzene revealed it to be crucial for chlorobenzene degradation. The chlorobenzene degradation pathway in strain GJ31 is thus a mosaic encoded by four gene clusters.

INTRODUCTION

Chlorinated aromatic compounds are only rarely synthesized in nature, but are produced widely through human activities in large quantities for various purposes. Vast amounts of these compounds have been deliberately or accidentally released into the biosphere. This has raised major environmental concerns because of the low rate of microbial degradation, resulting in the persistence of these compounds in the environment and accumulation in food chains (Schwarzenbach *et al.*, 2003; Wackett & Hershberger, 2001). However, some bacterial strains have evolved degradative pathways for chloroaromatics and can even use these compounds as growth substrates (Reineke, 2001; Wackett & Hershberger, 2001).

The aerobic microbial degradation of various chloroaromatic compounds occurs via chlorocatechols as central intermediates. These are usually further degraded through

†Present address: Sandoz, Basel, Switzerland.

‡Present address: Forschungszentrum Jülich GmbH, Geschäftsstelle BIO NRW, Düsseldorf, Germany.

Abbreviations: GST, glutathione *S*-transferase; 2-HMS, 2-hydroxy-5-formylpent-2,4-dienoate (2-hydroxymuconic semialdehyde); MFS, major facilitator superfamily.

The GenBank/EMBL/DDBJ accession numbers for the *nah* cluster, *mhp* cluster, *cbzE* cluster and upper pathway cluster sequences of *Pseudomonas putida* GJ31 are AY831460, AY831461, AY831462 and AY831463, respectively.

the modified *ortho*-cleavage pathway (Dorn & Knackmuss, 1978a, b; Kaschabek & Reineke, 1992; Schmidt & Knackmuss, 1980; Schmidt *et al.*, 1980). In contrast, *Pseudomonas putida* strain GJ31 has been shown to be able to degrade toluene and chlorobenzene simultaneously (Oldenhuis *et al.*, 1989), and rapidly degrades chlorobenzene via 3-chlorocatechol using a novel type of *meta*-cleavage pathway instead of the modified *ortho*-cleavage pathway (Mars *et al.*, 1997). The chlorocatechol 2,3-dioxygenase CbzE of strain GJ31 productively converts 3-chlorocatechol, and stoichiometric displacement of chloride leads to the production of 2-hydroxymuconate (Kaschabek *et al.*, 1998), which is further converted through the *meta*-cleavage pathway. The *cbzE* gene is localized on a 3103 bp *Pst*I fragment of the total DNA of strain GJ31. The gene cluster encoding a ferredoxin (CbzT) and part of a 2-hydroxymuconic semialdehyde dehydrogenase (CbzG) is preceded by a transposase gene, *tnpA1* (Mars *et al.*, 1999; Tropel *et al.*, 2002) (Fig. 1a), and has been suggested to be localized on a large plasmid (Mars *et al.*, 1999).

In this paper we describe the isolation of a large plasmid, pKW1, involved in chlorobenzene catabolism by *P. putida* GJ31. We report the cloning and sequence analysis of an overall length of 29.6 kb and identify the cluster(s) harbouring the genes that encode the *meta*-cleavage pathway for chlorocatechols in *P. putida* GJ31. The pathway is supported by demonstrating the functionality of key enzymes of the clusters and by transcription studies.

METHODS

Strains, plasmids, growth conditions and cell extracts. The strains and plasmids used in this study are summarized in Table 1. *Escherichia coli* DH5α clones were grown overnight in Luria–Bertani

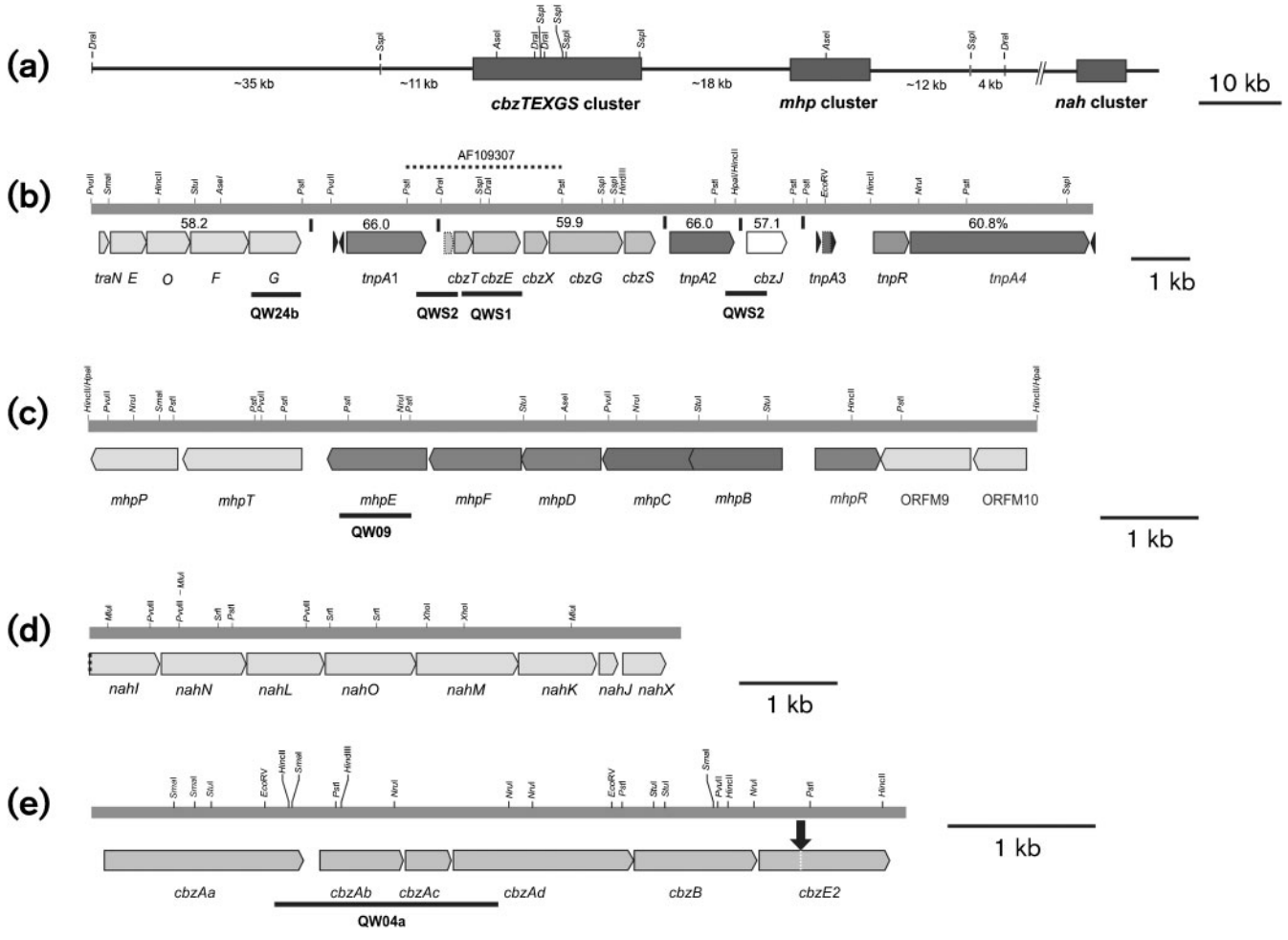

Fig. 1. Organization of the clusters encoded on plasmid pKW1 of *P. putida* strain GJ31 and the upper cluster on the chromosome. (a) Overall scheme of the 110 kb region on pKW1 comprising *cbzE*; (b) *cbzTEXGS* cluster, the dotted line indicates the region known from Mars *et al.* (1999), the G+C content is indicated for regions of the cluster defined by vertical black lines; (c) *mhp* cluster; (d) *nah* cluster; (e) upper chlorobenzene gene cluster localized on the chromosome. The arrow indicates the deletion at position 277 of *cbzE2*. The horizontal black lines show labelled probes used for hybridization.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype, phenotype or description*	Reference or source
Strains		
P. putida GJ31	Wild-type; toluene$^+$, chlorobenzene$^+$, *cbzE*	Oldenhuis *et al.* (1989)
Pseudomonas sp. 1dBTEX2	Benzene$^+$, catechol 2,3-dioxygenase subfamily I.2.A	Junca & Pieper (2004)
E. coli XL10	Tet^r Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lacI*^{q}ZΔM15 Tn*10* (Tet^r) Amy Cam^r]	Stratagene
E. coli DH5α	*supE*Δ*lacK169*(*ϕ80lacZ*ΔM15) *hsdR17 recA1 gyrA96 thi-1 relA1*	Hanahan (1983)
Plasmids		
pKW1	*cbzE*	GJ31; this study
pCR4Blunt-TOPO	Amp^r, cloning vector	Invitrogen
pJOE2702	ColE1, *bla*, P_{rhaBAD}, *rrnB*, expression vector	Volff *et al.* (1996)
pPCR bluescript SK+	ColE1 *f1 lacZ* Amp^r	Stratagene
pU2-36	*cbzE* in pJOE2702, chlorocatechol 2,3-dioxygenase	This study
pXPG	*cbzG* in pJOE2702, 2-hydroxymuconic semialdehyde dehydrogenase	This study
pXPJ	*cbzJ* in pJOE2702, 2-hydroxypent-2,4-dienoate hydratase	This study
pXPQ	*mhpF* in pJOE2702, acetaldehyde dehydrogenase	This study
pXPJ2	*mhpD* in pJOE2702, 2-hydroxypent-2,4-dienoate hydratase	This study
pXPF	*mhpC* in pJOE2702, 2-hydroxymuconic semialdehyde hydrolase	This study
pXPE2	*mhpB* in pJOE2702, 2,3-dihydroxyphenylpropionate 1,2-dioxygenase	This study

*Tet^r, Cam^r and Amp^r denote resistance to tetracycline, chloramphenicol and ampicillin, respectively. toluene$^+$, chlorobenzene$^+$ and benzene$^+$ indicate the capability of the strain to use the respective compound as sole carbon source.

medium containing 100 μg ampicillin ml^{-1} at 37 °C. The cells were harvested, resuspended in 0.1 M Tris/HCl (pH 7.5) containing 0.1 mM 1,4-dithiothreitol and disrupted using a French press (Aminco). The supernatant collected after centrifugation at 100 000 ***g*** was used as the enzyme extract. *P. putida* GJ31 was grown as previously described (Mars *et al.*, 1997).

Enzyme assays. All enzyme assays were performed in 50 mM phosphate buffer (pH 7.4) at 25 °C. Reactions were started by addition of cell extract (final concentration 0.02–0.2 mg protein ml^{-1}). One unit is defined as the activity required to convert one micromole of substrate or to form one micromole of product per minute under the conditions of the assay.

Catechol 2,3-dioxygenase activity was measured by determining product formation from catechol or 3-chlorocatechol at 375 or 290 nm, respectively ($\varepsilon_{2\text{-hydroxymuconic semialdehyde}}$=36 000 l mol^{-1} cm^{-1}; $\varepsilon_{2\text{-hydroxyhexa-2,4-dienedioate}}$=12 500 l mol^{-1} cm^{-1}; (Kaschabek *et al.*, 1998; Nozaki, 1970). 2,3-Dihydroxyphenylpropionate 1,2-dioxygenase activity was measured by determining product formation from 2,3-dihydroxyphenylpropionate at 394 nm ($\varepsilon_{2\text{-hydroxy-6-oxo-nona-2,4-diene 1,9-dicarboxylate}}$=19 150 l mol^{-1} cm^{-1}) (Bugg, 1993). 2-Hydroxymuconic semialdehyde hydrolase activity was measured by determining the NAD$^+$-independent decrease in concentration of 2-hydroxymuconic semialdehyde (0.06 mM) at 375 nm (Mars *et al.*, 1997). 2-Hydroxymuconic semialdehyde dehydrogenase activity was measured by determining the NAD$^+$-dependent decrease in concentration of 2-hydroxymuconic semialdehyde (0.06 mM) at 375 nm. NAD$^+$ was added to a final concentration of 0.5 mM. Oxalocrotonate decarboxylase activity was measured at 236 nm by determining the decrease in concentration of oxalocrotonate (0.1 mM) ($\varepsilon_{\text{oxalocrotonate}}$=6580 l mol^{-1} cm^{-1}) (Stanley *et al.*, 2000). 2-Hydroxypent-2,4-dienoate hydratase activity was measured by determining the decrease in concentration at 265 nm ($\varepsilon_{2\text{-hydroxypent-2,4-dienoate}}$=10 000 l mol^{-1} cm^{-1}) (Collinsworth *et al.*, 1973) in the presence of 0.33 mM $MgCl_2$. Acetaldehyde dehydrogenase (acylating) activity was measured by determining the coenzyme A-dependent NAD$^+$ reduction at 340 nm (Shingler *et al.*, 1992). Reaction mixtures contained 10 mM acetaldehyde, 100 mM coenzyme A and 0.28 mM NAD$^+$. Protein concentrations were determined using the Bradford reagent with BSA as the standard (Bradford, 1976).

Purification and general manipulations of DNA. Preparation of total DNA of *P. putida* GJ31, Southern blot hybridization, preparation of competent *E. coli* cells and subsequent transformations were performed as described elsewhere (Sambrook *et al.*, 1989). Plasmid DNA was isolated by the method of Wheatcroft & Williams (1981). Pure plasmid DNA was obtained by subsequent CsCl–ethidium bromide centrifugation. DNA restriction digests were performed according to the instructions of the manufacturers (Roche Diagnostics, New England Biolabs).

Cloning in *E. coli*. Genes were amplified from DNA of *P. putida* GJ31 using primers annealing upstream and downstream of the target genes, and including artificial *Nde*I and a *Bam*HI sites, respectively (see Table 2). Amplification was performed using Advantage cDNA polymerase mix (Clontech). After confirmation of the sequences, the fragments were cloned into the respective restriction sites of pJOE2702, and *E. coli* DH5α was transformed with the resultant plasmid.

PCR amplification. The reaction mixtures (50 μl) contained 50 pmol of each primer (see Table 2), 0.5 μg total or plasmid template DNA, 200 μM of each dNTP, 1 × PCR buffer (Stratagene), 5 % DMSO and 2.0 U Advantage DNA polymerase (Stratagene). PCR was performed with a touchdown thermocycle program: initial denaturation (98 °C, 1 min) before addition of the polymerase; 25–30 cycles with stepwise decreasing annealing temperature (60–50 °C; 30 s), polymerization (68 °C, 1 min per 1000 nt), and denaturation (98 °C, 45 s); with an additional 5 min of elongation during the last cycle. To obtain blunt-ended PCR products, Klenow fragment was

Table 2. Primers used in this study

Primer	Sequence (5′–3′)*	Amplicon size (bp)	Target/probe	Reference or source
Qno22	AGGGTTCGTTTCATCAACGAACGAG	1043	*cbzE*/probe QWS1	This study
Qno23	CAAGCTGATGAATCAAGACTTGGGG			
Qno24	ATCAGCGCGTCAGTTGGTAG	673	*tnpA1* downstream region/probe QWS2	This study
Qno25	TGCAGCAGACTCTCATCCGT			
Qno13	GGCGAGGCGAAGTTCTACATGGACC	2819	*cbzAaAbAcAd*/probe QW04	This study
Qno14	CATYTGCATBCGATGCCCRGCRATC			
Qno09	CGAGATCCAGCACATCCTGCGAGGCCACAA	1517	*cbzAaAbAcAd*/probe QW04a	This study
Qno10	TCCGCAGAGAGCGTGGTGCCATCATCCAGA			
Qno26	ARGTSACCCACGGYGAYGGC	733	*cbzK*/probe QW09	This study
Qno27	MRTAGACSCCSGCRTARCCC			
Qno32	CATGGACATGGCCCTGGCGGCGGCGAACTT	961	*traG*/probe QW24b	This study
Qno33	CGGGTCGTACAGGATTTCCAGCACTTCACG			
Kay4	CATGACATCCMCAAGGTSAC	684	*nahK* (fragment)	This study
Kay5	GCGCACSGTGATGTTGTCGC			
ESP1	GATACACATATGAGTATTATGAGAGTTGGC	970	*cbzE* (cloning in pJOE2702)	This study
ESEP3	CACTGGTCATTGGATCCATCATGTGTACAC			
cbzG-XP-1	TCAAGAGAGAGACATATGAAAGACATCAGG	1494	*cbzG* (cloning in pJOE2702)	This study
cbzG-XP-2	AATGGGGGCACAGGATCCTCAATAC			
cbzJ-XP-1	AGCCCCATATGACCCAGACGCCCAA	846	*cbzJ* (cloning in pJOE2702)	This study
cbzJ-XP-2	ACCGGGTTGATGTGCAGGGGATCCG			
mphF-XP-1	TTTCATATGACTACCAAACGTAAAGTTGCC	974	*mphF* (cloning in pJOE2702)	This study
mphF-XP-2	GAGCTTTTTACGGATCCTAACTGCGCT			
mphD-XP-3	TCACATATGAACACTTCACAAGAGACCCTT	849	*mphD* (cloning in pJOE2702)	This study
mphD-XP-4	AGCCAAGGATCCCAACTTTACGTTTGGTAG			
mphC-XP-1	TCCCATATGGATCGCAGGCTTCGGCTCTAT	959	*mphC* (cloning in pJOE2702)	This study
mphC-XP-2	TGTGAAGTGTCCATGCGGATCCTCGGTCAG			
mphB-XP-1	GTACATATGAACGCTTACCTACATTGCCTG	983	*mphB* (cloning in pJOE2702)	This study
mphB-XP-2	TGCAGTCACGATTAAGGATCCGTGAAGGTG			
NahOMod-1	GATCGAGGACAGCCATATGAGCAAGA	1001	*nahO* (cloning in pJOE2702)	This study
NahOMod-2	TCGCGCAGGATCCTGTCGTGCAGGGT			
Exdo-A-F	ATGAAVAAAGGHGTWHTGCGHCCMGG	421/716	*nahH* (fragment)	H. Junca, personal communication; Junca & Pieper (2004)
Exdo-A-R1	GYGGCCADGCYTCNGGRTTG			
Exdo-A-R719	TCGATVGAKGTRTCGGTCATG			
Exdo-A-F2	CATGACCGAYACMTCBATCG	1061	*nahHI* (fragment)	This study
NahI-R1	ACAGTCGGCGAAGACGATGC			
CbzERNAF	TGATGCGCCGACTCCC	262	*cbzE* (expression studies)	This study
CbzERNAR	AACTGCGCGACCCGCTTGA			
MphBRNAF	TTATTGGGCATTTCGACCCA	382	*mphB* (expression studies)	This study
MphBRNAR	CAGTTCACGAAAACAGGAAG			
MphERNAF	CATACCGGAAAGGCTGACAT	332	*mphE* (expression studies)	This study
MphERNAR	ATGTGTCGAAGCAGCACATC			
NahLRNAF	GAGGACGCCTACCACATCTC	250	*nahL* (expression studies)	This study
NahLRNAR	CCATCAGGTCCTTCTTGAGG			
bphAF668-3	GTTCCGTGTAACTGGAARTWYGC	535	*cbzAa* (expression studies)	Witzig *et al.* (2006)
bphAR1153-2	CCAGTTCTCGCCRTCRTCYTGHTC			
GltARNAF	GCCGATTTCATCCAGCATGGTC	200	*gltA* (expression studies)	P. Bielecki, personal communication
GltARNAR	TGGACCGGATCTTCATCCTCCA			

*Restriction sites are underlined.

incubated with the PCR reaction mixture for 30 min at 4 °C. Amplified template was purified with the Qiaquick PCR Purification kit (Qiagen).

PCR-based walking in DNA upstream and downstream from a known sequence was performed with the Universal Genome Walking kit and Advantage-GC cDNA PCR kit (BD Biosciences Clontech). Walks were extended by taking multiple steps using new primers based on the sequences obtained in previous steps. Primers to search for the presence of a catechol 2,3-dioxygenase of subfamily I.2.A (Eltis & Bolin, 1996) were designed based on a multiple sequence alignment of currently available members of this subfamily (Junca & Pieper, 2004). Primers were obtained from MWG-Biotech.

Extraction of mRNA, cDNA synthesis and RT-PCR. For gene expression studies, *P. putida* strain GJ31 was grown in mineral salt medium with chlorobenzene (corresponding to 5 mM, supplied via the vapour phase) as the sole carbon source. To assess constitutive expression, the strain was grown in parallel on fructose (5 mM). During exponential growth, 25 ml of each culture was mixed with the same volume of RNAprotect Bacteria reagent (Qiagen) and incubated for 5 min at room temperature, and cells were harvested by centrifugation. Harvested cells were resuspended in 100 µl water and immediately processed as previously described (Witzig *et al.*, 2007). cDNA was synthesized from 10 µg total RNA using SuperScript III reverse transcriptase (Invitrogen) according to the procedure from the manufacturer, followed by purification of cDNA using a Qiaquick PCR purification kit (Qiagen). RT-PCR was performed with 100 ng, 25 ng and serial dilutions thereof (twofold) as template using 35 cycles, an annealing temperature of 64 °C and (i) the primer set CbzERNAF/CbzERNAR (annealing temperature 64 °C) to amplify a 262 bp *cbzE* gene fragment, (ii) the primer set MphBRNAF/MphBRNAR to amplify a 382 bp *mphB* gene fragment, (iii) the primer set MphERNAF/MphERNAR to amplify a 332 bp *mphE* gene fragment, (iv) the primer set NahLRNAF/NahLRNAR to amplify a 250 bp *nahL* gene fragment, (v) the primer set bphA F668-3/bphA R1153-2 (Witzig *et al.*, 2006) to amplify a 535 bp *cbzAa* gene fragment, and (vi) the primer set GltARNAF/GltARNAR to amplify a 200 bp *gltA* gene fragment. Amplification products were separated on 1 % agarose gels and stained with ethidium bromide.

DNA sequence analysis. DNA sequencing was performed by MWG-Biotech, Ebersberg, Germany. Assembled contigs were used for DNA and protein similarity searches in GenBank databases performed with the BLASTN and BLASTP programs of the National Center for Biotechnology Information website (http://ncbi.nlm.nih.gov). Translated protein sequences were aligned with CLUSTAL_X 1.83 using default values. Phylogenetic trees were constructed with MEGA4 (Tamura *et al.*, 2007) using the Neighbour-Joining (N-J) algorithm (Saitou & Nei, 1987) with p-distance correction and pairwise deletion of gaps and missing data. A total of 100 bootstrap replications were done to test for branch robustness.

Chemicals. Catechol derivatives were obtained from our laboratory stock and were purified by sublimation prior to use. 2-Hydroxy-5-formylpent-2,4-dienoate (2-hydroxymuconic semialdehyde; 2-HMS) was prepared *in situ* by incubation of a solution containing 0.1 mM catechol in 45 mM phosphate buffer (pH 7.4) with heat-treated cell extract of *m*-toluate-grown *P. putida* mt-2. 2-Hydroxymuconate was prepared as previously described (Kaschabek *et al.*, 1998) and further converted due to spontaneous reaction in buffer at pH 7.4 to give oxalocrotonate. 2-Hydroxypent-2,4-dienoate was prepared from DL-allylglycine using L-amino acid oxidase, as described by Collinsworth *et al.* (1973). 2,3-Dihydroxyphenylpropionate was obtained from Professor Timothy D. H. Bugg, University of Warwick.

RESULTS

The chlorocatechol gene cluster is localized on plasmid pKW1

In order to obtain information on the localization of the chlorocatechol gene cluster of *P. putida* GJ31 with the *cbzE* gene encoding chlorocatechol 2,3-dioxygenase as the key gene, identification of plasmids was performed. The presence of a single plasmid, termed pKW1, could be demonstrated, with a size of 180 ± 10 kb as determined by digestion with a range of restriction enzymes. Hybridization of a 1 kb *cbzE* gene fragment (QWS1) harbouring a *Dra*I restriction site, with two *Dra*I/*Ssp*I fragments (0.7 and 2.3 kb) of the plasmid, confirmed that *cbzE* is localized on plasmid pKW1.

The gene regions flanking *cbzE* comprise transfer genes and transposable elements

Sequences surrounding the previously described 3103 bp *Pst*I fragment comprising the *cbzE* gene (Mars *et al.*, 1999) (see Fig. 1a) were obtained by genome walking. Successive cloning and sequencing allowed the isolation of 6.3 kb upstream of the previously described *tnpA1* and 10.9 kb downstream of *cbzG*.

The predicted amino acid sequences of *traN*, *traE*, *traO*, *traF* and *traG* (Fig. 1b) show remarkably high sequence similarity to Tra proteins (Table 3), which are responsible for mobilization and transfer of DNA (Pohlman *et al.*, 1994). A sequence of 262 bp identical to the 'ΔxIS element' of plasmid pAM10.6 of *Pseudomonas fluorescens* strain Cb36 (Peters *et al.*, 1997) follows *traG*. This sequence is flanked by two inverted repeats, IR1 'left' and IR1 'right' (38 nt in length, 36 identical, positions 4814–4851 and 5038–5075), typical of the Tn*3* family of elements (Sherratt, 1989) (Fig. 2). The next ORF downstream, termed *tnpA1*, resembled transposase-encoding genes and is identical in amino acid sequence to the corresponding deduced gene product of *Acidovorax* sp. JS42 (YP_974115).

The following sequence stretch has been analysed previously (Mars *et al.*, 1999) and comprises *cbzT*, *cbzE* and *cbzX*, encoding ferredoxin reductase, chlorocatechol 2,3-dioxygenase and a protein of unknown function, respectively.

The deduced amino acid sequence (486 aa) of *cbzG* shows significant sequence similarities to 2-hydroxymuconic semialdehyde dehydrogenases, particularly to those of strains documented to possess a catechol 2,3-dioxygenase of subfamily I.2.C (Eltis & Bolin, 1996), which is also the subfamily to which CbzE belongs. Operons that contain a gene encoding a subfamily I.2.C catechol 2,3-dioxygenase, such as that of *Cupriavidus necator* JMP134 (Fig. 2), usually have a quite conserved gene structure of ORFs encoding a ferredoxin, a catechol 2,3-dioxygenase, an unknown protein and a 2-hydroxymuconic semialdehyde dehydrogenase. Cell extracts of *E. coli* (pXPG) overexpressing *cbzG*

Table 3. ORFs identified in *P. putida* GJ31

ND, Not determined: gene incomplete.

Gene			Gene product			Closest homologue		
ORF	**Coordinates (5′–3′)**	**G+C content (%)**	**Number of residues**	**Molecular mass (kDa)**	**Function**	**Sequence identity (%)**	**Organism**	**Accession number**
pKW1: *cbzTEXGS* cluster								
traN	203–394	58.3	63	6.48	Transfer protein	58	*Salmonella typhimurium*	NP_511194
traE	391–1113	57.7	240	26.98	DNA topoisomerase III	46	*Salmonella typhimurium*	NP_511195
traO	1113–1991	56.0	292	32.53	Transfer protein	49	*Salmonella typhimurium*	NP_511196
traF	1988–3145	59.8	385	41.29	Transfer protein	44	*Salmonella typhimurium*	NP_511197
traG	3157–4188	59.5	343	38.09	Transfer protein	86	*P. putida* H	AF130439
tnpA1	5101–6681	66.0	526	57.98	Transposase	99	*Acidovorax* sp. JS42	YP974115
cbzT	7246–7605	58.6	120	12.75	Ferredoxin	100	*P. fluorescens* SK1	AAP51202
*cbzE**	7617–8561	55.7	315	34.95	Chlorocatechol 2,3-dioxygenase	97	*P. fluorescens* SK1	AAP51203
cbzX	8642–9106	64.7	155	15.72	Unknown	100	*P. fluorescens* SK1	AAP51204
*cbzG**	9144–10 604	58.8	486	52.45	2-HMS dehydrogenase	100	*P. fluorescens* SK1	AAP51205
cbzS	10 644–11 252	55.7	204	22.08	GST	100	*Pseudomonas* sp. CT14	YP_001966313
tnpA2	11 556–12 833	66.0	425 (526)†	54.49	Transposase	99	*Acidovorax* sp. JS42	YP974115
*cbzJ**	13 085–13 879	57.1	264	28.51	2-Hydroxypent-2,4-dienoate hydratase	99	*Acidovorax* sp. JS42	YP974116
tnpA3	14 595–14 753		106		Transposase fragment			
tnpR	16 713–17 312	65.4	200	21.88	Resolvase	94	*Pseudomonas* sp. ND6	NP_943127
tnpA4	17 296–20 325	60.7	1009	115.37	Transposase	99	*Pseudomonas* sp. CT14	YP_001966298
pKW1: *mhp* cluster								
mhpP	881–3	54.7	293 (427)†	32.62	Membrane porin OprD family	58	*Azotobacter vinelandii* AvOP	ZP_00419203
mhpT	2142–926	56.6	403	41.81	MFS family transporter protein	61	*Azotobacter vinelandii* AvOP	ZP_00416222
mhpE	3422–2412	57.4	336	36.16	4-Hydroxy-2-oxovalerate aldolase	88	*P. putida* W619	YP_001748854
*mhpF**	4372–3434	56.4	312	32.71	Acetaldehyde dehydrogenase	84	*P. putida* PaW630	AAL83664
*mhpD**	5181–4369	71.1	270	28.07	2-Hydroxypent-2,4-dienoate hydratase	77	*P. putida* W619	YP_001748852
*mhpC**	5940–5194	55.2	248	27.59	2-HMS hydrolase	82	*P. putida* W619	YP_001748851
*mhpB**	7121–6075	58.1	315	34.53	2,3-Dihydroxyphenylpropionate 1,2-dioxygenase	95	*P. putida* ML2	AAG09232
mhpR	7358–8023	52.6	221	24.52	GntR family regulator	53	*Dechloromonas aromatica* RCB	YP_284124
ORFM9	8935–8018	51.4	305	33.76	Unknown	53	*Marinobacter algicola* D6839	ZP_01895523
ORFM10	9503–8964	53.3	179	19.30	Unknown	48	*Rhizobium* sp. NGR234	AAQ87234
pKW1: *nah* cluster								
nahI	1–711	66.9	236 (486)†	ND	2-HMS dehydrogenase	99	*P. stutzeri* OM1	BAA31265
nahN	723–1586	67.4	288	31.90	2-HMS hydrolase	96	*P. stutzeri* AN10	AAD02150
nahL	1583–2368	65.4	261	27.90	2-Hydroxypent-2,4-dienoate hydratase	97	*P. putida* MT53	YP_709325
nahO	2383–3306	65.4	308	33.02	Acetaldehyde dehydrogenase	99	*P. stutzeri* AN10	AAD021520
nahM	3318–4358	65.3	347	37.27	4-Hydroxy-2-oxovalerate aldolase	100	*P. stutzeri* AN10	AAD02153
nahK	4355–5149	62.5	264	28.56	4-Oxalocrotonate decarboxylase	97	*P. stutzeri* AN10	AAD02154

Table 3. cont.

ORF	Gene		Gene product			Closest homologue		
	Coordinates (5′–3′)	G+C content (%)	Number of residues	Molecular mass (kDa)	Function	Sequence identity (%)	Organism	Accession number
nahJ	5198–5389	65.1	63	6.84	4-Oxalocrotonate tautomerase	95	*P. stutzeri* AN10	AAD02155
nahX	5420–5860	64.7	147	15.10	Unknown	67	*Pseudomonas* sp. KB35B	ABB71216
Chromosome								
cbzAa	80–1432	60.2	450	51.04	Benzene dioxygenase, α-subunit	99	*P. putida* F1	AAA26005
cbzAb	1543–2106	58.5	187	22.02	Benzene dioxygenase, β-subunit	100	*P. putida* F1	AAA26006
cbzAc	2115–2438	56.8	107	11.89	Ferredoxin	100	*P. putida* F1	AAA26007
cbzAd	2438–3670	63.7	410	42.91	NADH : ferredoxin oxidoreductase	99	*P. putida* F1	AAA26008
cbzB	3667–4494	60.3	275	28.80	Benzene dihydrodiol dehydrogenase	99	*P. putida* F1	AAA26009
cbzE2	4510–5384	58.3	Frame shift	ND	Catechol 2,3-dioxygenase	95	*P. putida* F1	AAA26010

*Respective enzyme activity verified.
†Length of complete enzyme is given in parentheses.

showed a strong 2-hydroxymuconic semialdehyde dehydrogenase activity of 1800 units (g protein)$^{-1}$, which was absent in *E. coli* (pJOE2702), indicating that *cbzG* encodes a functional 2-hydroxymuconic semialdehyde dehydrogenase.

The deduced 204 aa protein encoded by *cbzS* is similar to BphK, a glutathione *S*-transferase (GST) that occurs in some biphenyl pathways such as those of *Burkholderia xenovorans* strain LB400 and *Pseudomonas pseudoalcaligenes* strain KF707 (69–70 % amino acid identity) (Bartels *et al.*, 1999). The catabolic genes are followed by *tnpA2*, identical in sequence to *tnpA1*; however, the initial 140 nt of *tnpA1* were absent in *tnpA2*.

The protein encoded by *cbzJ* shows sequence similarities to 4-oxalocrotonate decarboxylases (31 % sequence identity with XylI of *P. putida* mt-2, CAC86800), but also to 2-hydroxypent-2,4-dienoate hydratases (35 % sequence identity with XylJ of *P. putida* mt-2, P23107), enzymes catalysing subsequent reactions in catechol *meta*-cleavage pathways, but sharing significant amino acid similarities (see Fig. 3). For CbzJ, a high similarity was observed only to the non-characterized proteins of *Acidovorax* sp. JS42 (YP_974116) and *Pseudomonas* sp. CT14 (ABA25976), or the truncated proteins of *Comamonas* sp. CNB1 (ABB13584) and *Delftia* sp. AN3 (ABI20724). Phylogenetic analyses (Fig. 3) show that these proteins constitute a novel branch in the phylogeny of members of the tautomerase superfamily (Poelarends *et al.*, 2008). A significant 2-hydroxypent-2,4-dienoate hydratase activity of 1100 units (g protein)$^{-1}$ and the absence of 4-oxalocrotonate decarboxylase activity in cell extracts of *E. coli* (pXPJ) prove that CbzJ functions as a 2-hydroxypent-2,4-dienoate hydratase.

A sequence of 106 bp of the terminal repeat of part of IS*1071* was found upstream of a truncated *tnpA3* (Fig. 2), the predicted encoded peptide being identical with the N-terminal portion of TnpA of IS*1071* of *Pseudomonas* sp. strain H and *Comamonas testosteroni* strain BR60 (Providenti *et al.*, 2006; Sota *et al.*, 2006). At 159 bp downstream of the *tnpA3* start codon, an inverted repeat of 38 nt (IR2 'left'), identical to that of transposon Tn*5501* (Lauf *et al.*, 1998) (28 % identical nucleotides to IS1 'left'), disrupts the *tnpA3* gene. The second repeat (IR2 'right') (26 % identical nucleotides to IS1 'right') was observed about 5.5 kb downstream. The complete sequence stretch between these repeats on pKW1 was highly similar (97 % nucleotide sequence identity) to that observed on plasmid pPGH1 of *P. putida* strain H (Lauf *et al.*, 1998), probably encoding a resolvase (TnpR, 91 % amino acid identity) and a transposase (TnpA4, 88 % amino acid sequence identity) of the Tn*3* family of transposases.

A gene region similar to the above-mentioned *tnpA1*·*cbzTEXGS*·*tnpA2*·*cbzJ*·*tnpA3* gene module has also been observed on plasmid pCT14 of *Pseudomonas* sp. strain CT14 (Bramucci *et al.*, 2006). In contrast to pKW1, the plasmid pCT14 lacks most of the genes (except *traJ* and *traI*) necessary for self-mobilization.

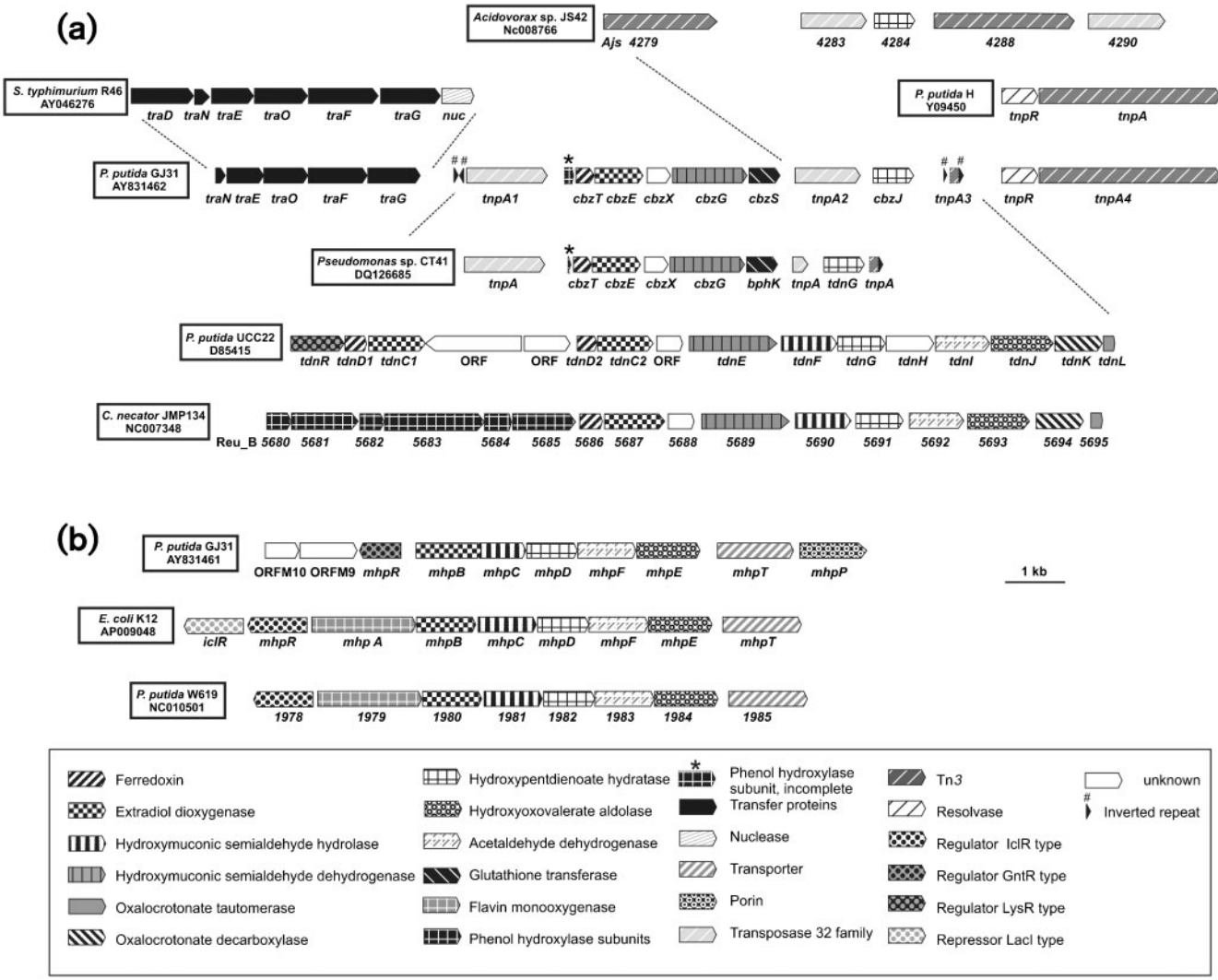

Fig. 2. Organization of the *cbzTEXGS* cluster (a) and the *mhp* gene cluster (b) in comparison with related gene clusters.

Plasmid pKW1 harbours a 2,3-dihydroxyphenylpropionate gene cluster

Only some of the *meta*-pathway enzymes, which are necessary for 3-chlorocatechol mineralization, were encoded on the region of plasmid pKW1 described above, and those encoding oxalocrotonate tautomerase, oxalocrotonate decarboxylase, 4-hydroxy-2-oxovalerate aldolase and acetaldehyde dehydrogenase were absent (Fig. 4). The use of primers Qno26 and Qno27 targeting conserved regions of 4-hydroxy-2-oxovalerate aldolases encoded in 2,3-dihydroxyphenylpropionate *meta*-cleavage pathway clusters of *E. coli* allowed the amplification of a 730 bp fragment from plasmid DNA. The deduced amino acid sequence showed 84 % identity with 4-hydroxy-2-oxovalerate aldolase of *E. coli* strain K-12 (Ferrandez *et al.*, 1997) and the respective ORF was termed *mhpE*. A total of 2.8 kb downstream and 6.1 kb upstream of the *mhpE* gene were analysed by genome walking. DNA sequence analysis of the 9632 bp region showed nine ORFs plus an incomplete one. The encoded proteins showed high similarity to proteins of the 2,3-dihydroxyphenylpropionate *meta*-cleavage pathway (Diaz *et al.*, 2001), and the gene organization resembled that observed in *E. coli* strain K-12 (Ferrandez *et al.*, 1997) or *P. putida* strain W619 (NC_010501).

Overall, five catabolic genes (*mhpB*, *C*, *D*, *F* and *E*) could be identified (Fig. 1c). *mhpB* encodes a polypeptide (315 aa) which shows significant sequence similarities to enzymes with a validated function as 2,3-dihydroxyphenylpropionate 1,2-dioxygenases (Diaz *et al.*, 2001). 2,3-Dihydroxyphenylpropionate 1,2-dioxygenases belong to the LigB superfamily of extradiol dioxygenases and show a broad specificity with catechol and methylcatechols as accepted substrates (Barnes *et al.*, 1997; Diaz *et al.*, 2001). Enzyme assays in cell extracts of *E. coli* (pXPE2) showed strong catechol 2,3-dioxygenase activity [1830 units (g protein)$^{-1}$] as compared with those of the controls.

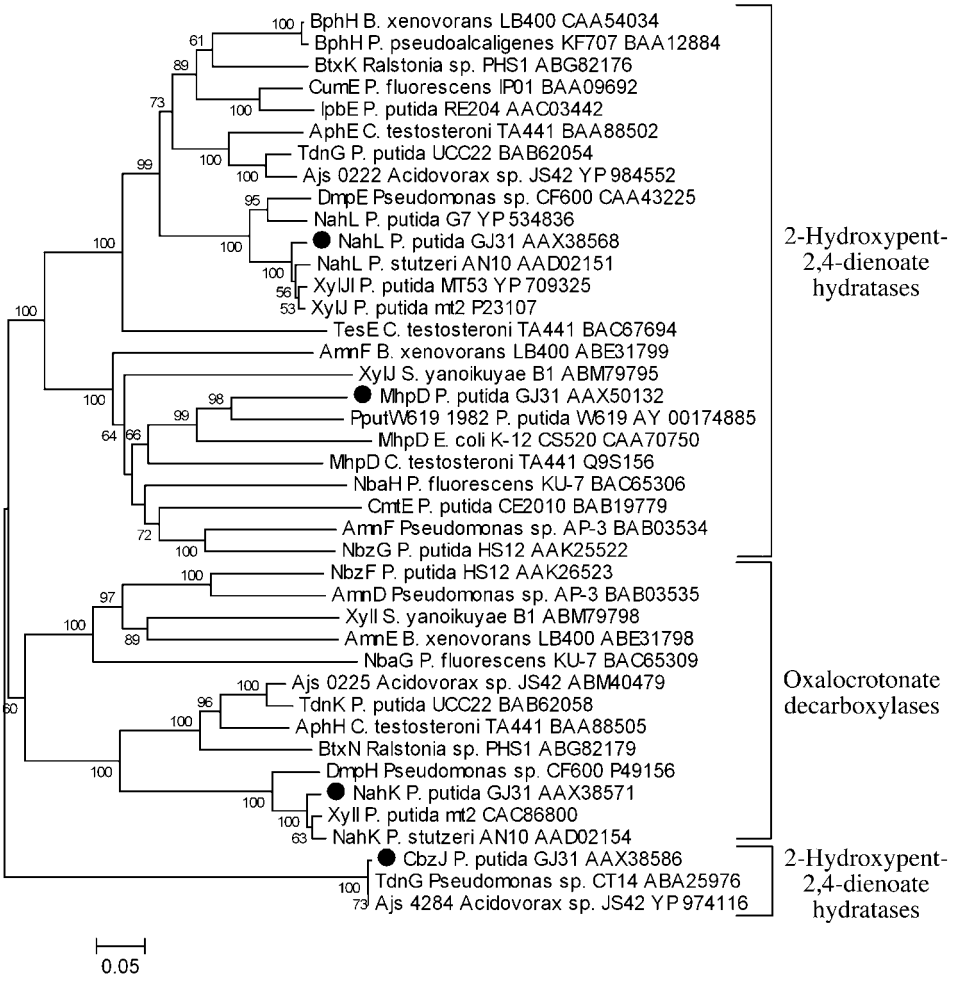

Fig. 3. Evolutionary relationships of 2-hydroxypent-2,4-dienoate hydratases and oxalocrotonate decarboxylases. The evolutionary histories were inferred using the neighbour-joining method and the p-distance model. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007). Bootstrap values above 50 % from 100 neighbour-joining trees are indicated to the left of the nodes. Bar, 0.05 amino acid differences per site; ●, proteins of *P. putida* GJ31 identified in this study.

However, activity with 2,3-dihydroxyphenylpropionate was significantly higher (1.8-fold) than that with catechol, in accordance with the putative function of the protein. In contrast, activity of CbzE with 2,3-dihydroxyphenylpropionate was only 20 % of that with catechol, as expected for a (chloro)catechol 2,3-dioxygenase. Like most catechol 2,3-dioxygenases so far described, MhpB failed to convert 3-chlorocatechol.

In accordance with the assumed function of the gene cluster as having evolved for the degradation of 2,3-dihydroxyphenylpropionate, the protein (308 aa) encoded by *mhpC* showed highest similarities with 2-hydroxy-6-ketonona-2,4-dienoate hydrolases such as MphC of *E. coli* K-12 (73 %) (Ferrandez *et al.*, 1997). Strong 2-hydroxymuconic semialdehyde hydrolase activity [1100 units (g protein)$^{-1}$] in *E. coli* (pXPF) expressing MhpC in comparison with the control proves *mhpC* to encode a functional hydrolase.

The deduced amino acid sequence (270 aa) of MhpD showed significant sequence similarities to the protein family of 4-oxalocrotonate hydratases/decarboxylases. High similarity was observed with MhpD 2-hydroxypent-2,4-dienoate hydratases such as that of *E. coli* K-12 (66 %) (Ferrandez *et al.*, 1997) (Fig. 3). Enzyme assays with crude extracts of *E. coli* (pXPJ2) expressing MhpD confirmed this enzyme to encode a hydratase, as evidenced by a strong activity [840 units (g protein)$^{-1}$] with 2-hydroxypent-2,4-dienoate and the absence of a 4-oxalocrotonate decarboxylase activity.

mhpF encodes a protein of 312 aa sharing up to 84 % identical residues with a previously reported acetaldehyde

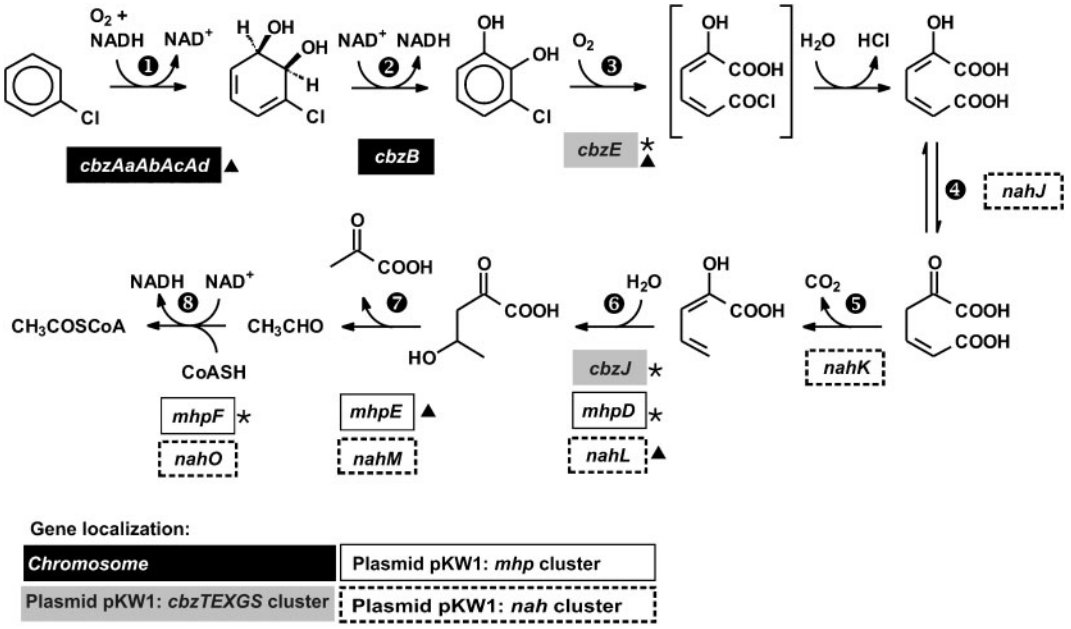

Fig. 4. Degradative pathway for chlorobenzene in *P. putida* strain GJ31. Enzymes involved are: 1, chlorobenzene dioxygenase; 2, chlorobenzene dihydrodiol dehydrogenase; 3, extradiol dioxygenase; 4, oxalocrotonate tautomerase; 5, oxalocrotonate decarboxylase; 6, 2-hydroxypent-2,4-dienoate hydratase; 7, 4-hydroxy-2-oxovalerate aldolase; 8, acetaldehyde dehydrogenase (acetylating). Asterisks indicate activity detected with an expression clone; ▲, RT-PCR determination of induction in the presence of chlorobenzene.

dehydrogenase integrated in proteobacterial *meta*-cleavage pathways (Yano *et al.*, 2007). Analysis of cell extracts of *E. coli* (pXPQ) confirmed *mhpF* to encode an acetaldehyde dehydrogenase, as evidenced by an activity of 350 units (g protein)$^{-1}$, which was absent in the control lacking the *mhpF* insert. Also, the protein (336 aa) encoded by *mhpE* was most closely related to proteins of 2,3-dihydroxyphenylpropionate pathways and showed up to 88 % identity with aldolases cleaving 4-hydroxy-2-oxovalerate to give pyruvate and acetaldehyde (Shingler *et al.*, 1992). *mhpR*, located 339 bp upstream of *mhpB*, is the only ORF transcribed in a direction opposite to the other genes of this cluster. Sequence comparisons indicate that MhpR belongs to the GntR family of transcriptional regulators, and significant similarity (37 % identity) was observed with the OhpR regulator of the 2,3-dihydroxyphenylpropionate catabolic operon of *Rhodococcus* sp. strain V49 (Powell & Archer, 1998). *mhpT* (403 aa) encodes a protein of the major facilitator superfamily (MFS), and the deduced protein sequence shows high similarity (>50 % identity) to MhpT proteins assumed to be 3-hydroxypropionate transporters (e.g. P77589) and in *Enterobacteriaceae* typically encoded upstream of the 2,3-dihydroxyphenylpropionate pathway genes (e.g. AP009048). A genetic organization similar to the one observed here is also suggested to be present in *P. putida* W619 (NC_010501).

The deduced amino acid sequence (293 aa) of the incomplete *mhpP* showed significant sequence similarities to porins of the Opr family, specifically to PaaM, which has been suggested to be involved in the uptake of phenylacetate by *P. putida* U (45 % amino acid identity) (Olivera *et al.*, 1998). Similar proteins are usually not encoded in 2,3-dihydroxyphenylpropionate catabolic gene clusters. Two ORFs were identified preceding the 2,3-dihydroxyphenylpropionate catabolic gene cluster (Table 3). However, their functions remain unknown.

Hybridization with probe QW09 (targeting *mhpE*) and probe QWS1 (targeting *cbzE*) showed both genes to be localized on a single 39 kb *Ase*I fragment (Fig. 1). As probe QW09 also hybridized with a 21 kb *Ase*I/*Ssp*I fragment, *mhpE* is located on the part of the *mhp* cluster near the *cbzE* cluster, and all genes but *mhpR* are oriented in the opposite direction to the *cbzE* cluster.

Plasmid pKW1 harbours an incomplete *nah* cluster

Genes encoding 4-oxalocrotonate tautomerase and 4-oxalocrotonate decarboxylase, necessary for the conversion of 2-hydroxymuconate, the 3-chlorocatechol ring-cleavage product, to 2-hydroxypent-2,4-dienoate, were absent in both of the aforementioned gene clusters (see Fig. 4). To search for respective genes, conserved regions of oxalocrotonate decarboxylase-encoding genes were used for the design of the primer pair Kay4/Kay5 (see Table 2). A 0.79 kb PCR fragment obtained with pKW1 DNA as

template was sequenced, and a total of 5943 bp of sequence framing this fragment was analysed after PCR-based walking.

The analysed gene region (Fig. 1d) showed high identity (96 %) over the whole sequence to the region encoding NahINLOMKJ of the *nahGTHINLOMKJ* gene cluster of *Pseudomonas stutzeri* AN10 (Bosch *et al.*, 2000). As the protein sequence differences did not involve amino acids assumed to be crucial for enzyme activity, the analysed region can be assumed to encode the following: NahI, 2-hydroxymuconic semialdehyde dehydrogenase (incomplete); NahN, 2-hydroxymuconic semialdehyde hydrolase; NahL, 2-hydroxypent-2,4-dienoate hydratase; NahO, acetaldehyde dehydrogenase (acylating); NahM, 4-hydroxy-2-oxovalerate aldolase; NahK, oxalocrotonate decarboxylase; and NahJ, oxalocrotonate tautomerase. The function of NahX (147 aa) remains unknown. Screening upstream of *nahI* using primers Exdo-A-F2/NahI-R1 (Table 2), designed based on a multiple sequence alignment of currently available subfamily I.2.A catechol 2,3-dioxygenases and the available *nahI* sequence of GJ31, gave no indication of the presence of a NahH catechol 2,3-dioxygenase-encoding gene. However, a PCR product of the expected 1061 bp was observed using total DNA of *Pseudomonas* sp. 1dBTEX2 (Junca & Pieper, 2004) as the control. Similarly, PCR products of the expected length (421 and 716 bp) were observed with primer pairs Exdo-A-F/Exdo-A-R1 and Exdo-A-F/Exdo-A-R719, respectively, using only *Pseudomonas* sp. 1dBTEX2 DNA as the template, indicating the absence of a gene encoding a subfamily I.2.A catechol 2,3-dioxygenase from the genome of *P. putida* GJ31.

The upper pathway genes are located on the chromosome

The degradation of chlorobenzenes in strains employing an *ortho*-cleavage pathway is typically initiated by a Rieske non-haem iron oxygenase of the toluene/biphenyl subfamily. Primers Qno13 and Qno14, targeting conserved regions in the toluene/biphenyl oxygenase, amplified a 2.8 kb fragment termed QW04, when total DNA of strain GJ31 was used as template. The fragment was sequenced and gene walking applied to obtain a total of 5493 bp of sequence information framing QW04.

The analysed gene region (Fig. 1e) showed 99 % sequence identity to the TodC1C2BADE-encoding gene region of *P. putida* F1 (Zylstra & Gibson, 1989). Accordingly, a total of six ORFs, termed *cbzAa*, *cbzAb*, *cbzAc*, *cbzAd*, *cbzB* and *cbzE2*, could be identified, with the first four clearly encoding the α- and β-subunits, ferredoxin and NADH : ferredoxin oxidoreductase of chlorobenzene dioxygenase, respectively. Whereas CbzAb and CbzB were identical to TodC2 and TodB, respectively, CbzAa, CbzAd and CbzD differed from the orthologous Tod proteins by only a single amino acid each. As these differences are localized in regions not crucial for protein performance (Friemann *et al.*, 2009) or constitute conservative amino acid differences (Hülsmeyer *et al.*, 1998), it can be assumed that all encoded proteins are functional and similar in substrate specificity to those described from strain F1 and are capable of transforming chlorobenzene to 3-chlorocatechol (Gibson *et al.*, 1968) sufficiently to allow growth on chlorobenzene (Ravatn *et al.*, 1998) (see Fig. 4). CbzE2, like TecE of *Ralstonia* sp. PS12 (Beil *et al.*, 1999), shows a one-base deletion at position 277 compared with the *todE* sequence, probably resulting in an inactive enzyme. Probe QW04a targeting chlorobenzene dioxygenase-encoding genes failed to hybridize with DNA of plasmid pKW1, the single plasmid in strain GJ31, confirming the upper pathway operon to be located on the chromosome.

Transcription of pathway segments

To analyse whether genes of the above-described gene clusters were transcribed in response to chlorobenzene, RT-PCR experiments were performed with total RNA extracted from strain GJ31 growing on fructose or chlorobenzene using primers targeting *cbzAa*, *cbzE*, *mhpB*, *mhpE* and *nahL*. While transcripts of *nahL* and *cbzE* were, like the housekeeping gene *gltA* encoding citrate synthase (Wehmhöner *et al.*, 2003), detectable after growth on fructose in amounts of less than 10 ng cDNA, transcripts of *cbzAa* as well as of *mhpB* and *mhpE* were only detectable at 100 ng of cDNA template. More importantly, with regard to total RNA, transcript levels of *cbzE* and *cbzAa* in chlorobenzene-grown cells were increased by more than two orders of magnitude compared with fructose-grown cells, and those of *mhpB*, *mhpE* and *nahL* by more than one order of magnitude. When the relative levels of transcripts were normalized with respect to the level of *gltA* transcripts, it was evident that the levels of both the *cbzE* and the *cbzAa* transcripts were significantly increased, but those of genes within the *nah* and *mhp* gene cluster only increased by two- to fourfold (Table 4).

DISCUSSION

The aerobic degradation of chlorobenzenes, in all strains characterized in this aspect so far, is initiated by a Rieske non-haem iron oxygenase of the toluene/biphenyl family (Gibson & Parales, 2000; Reineke, 1998; Witzig *et al.*, 2006). These enzymes are typically of relatively broad substrate specificity (Gibson & Parales, 2000) and, as an example, toluene dioxygenase of *P. putida* F1 accepts, besides toluene, also chlorobenzene, *p*-chlorotoluene and 1,2-dichlorobenzene as substrates (Beil *et al.*, 1998; Gibson *et al.*, 1968).

The *cbzAaAbAcAdBE2* cluster of strain GJ31, encoding chlorobenzene dioxygenase, dihydrodiol dehydrogenase and catechol 2,3-dioxygenase is of a structure identical to those of chlorobenzene upper pathway catabolic operons, such as those identified in *Ralstonia* sp. PS12 (Beil *et al.*, 1997) and *Acidovorax* sp. P51 (van der Meer *et al.*, 1991),

Table 4. Transcription of catabolic genes in fructose- and chlorobenzene-grown cells of *P. putida* GJ31 as determined by RT-PCR

Values represent *n*-fold change in the ratio of transcript between the target gene and the reference gene (*gltA*) compared with non-inducing conditions.

Gene	Carbon source	PCR template amount (cDNA)												Fold induced by chlorobenzene
		100 ng	25 ng	12.5 ng	6.3 ng	3.1 ng	1.6 ng	781 pg	391 pg	195 pg	98 pg	49 pg	24.4 pg	12.2 pg	6.1 pg	
cbzAa	Fructose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	>32
	Chlorobenzene	+	+	+	+	+	+	+	+	−	−	−	−	−	−	
cbzE	Fructose	+	+	+	+	+	−	−	−	−	−	−	−	−	−	32
	Chlorobenzene	+	+	+	+	+	+	+	+	+	+	+	+	+	−	
mhpB	Fructose	+	−	−	−	−	−	−	−	−	−	−	−	−	−	4
	Chlorobenzene	+	+	+	+	+	−	−	−	−	−	−	−	−	−	
mhpE	Fructose	+	−	−	−	−	−	−	−	−	−	−	−	−	−	2
	Chlorobenzene	+	+	+	+	−	−	−	−	−	−	−	−	−	−	
nahL	Fructose	+	+	+	+	−	−	−	−	−	−	−	−	−	−	4
	Chlorobenzene	+	+	+	+	+	+	+	+	+	−	−	−	−	−	
gltA	Fructose	+	+	+	+	+	+	−	−	−	−	−	−	−	−	1
	Chlorobenzene	+	+	+	+	+	+	+	+	+	−	−	−	−	−	

and nearly identical in sequence (5478/5494 nt) to the *todC1C2BADE* cluster of *P. putida* strain F1 growing with toluene via a *meta*-cleavage pathway (Mosqueda *et al.*, 1999; Zylstra & Gibson, 1989), and the high level of transcription in response to chlorobenzene validated its involvement in chlorobenzene degradation by strain GJ31. Typically, in chlorobenzene degraders, the respective upper pathway gene clusters are combined with a chlorocatechol gene cluster so that degradation can proceed via chlorocatechols and a chlorocatechol *ortho*-cleavage pathway. As an example, the events of the evolution of a chlorobenzene pathway in *Ralstonia* sp. JS705 have been traced as the recombination of two pathway segments from two different ancestors to form one large catabolic gene region (Müller *et al.*, 2003). The high mobility of upper pathway genes is further evidenced by the fact that the upper pathway genes on plasmid pP51 of *Acidovorax* sp. strain P51 (van der Meer *et al.*, 1991) and in *Ralstonia* sp. strain PS12 (Beil *et al.*, 1999; Müller *et al.*, 2003) are carried on transposons, or are localized on genomic islands, as in *Bordetella petrii* (Gross *et al.*, 2008). Whether the upper pathway genes of strain GJ31 belong to its core genome or have been recruited by horizontal gene transfer remains to be elucidated.

In strains degrading toluene via initial dioxygenation, such as *P. putida* F1 and *P. putida* DOT-T1, a complete hydrolytic branch of a *meta*-cleavage pathway is encoded by the same pathway gene cluster that encodes enzymes for initial dioxygenation and dehydrogenation (Lau *et al.*, 1994; Menn *et al.*, 1991; Mosqueda *et al.*, 1999). However, chloroaromatic degraders have been reported to avoid the presence of an active extradiol dioxygenase. In the case of strain P51, point mutations leading to a frameshift, or the introduction of stop codons in the case of strain PS12, have caused inactivation of the disadvantageous *meta*-cleavage genes for chlorobenzene degradation (Beil *et al.*, 1999). Such a defect in the catechol 2,3-dioxygenase is also found in CbzE2 of strain GJ31, indicating that the chromosomally encoded upper pathway evolved for chlorobenzene degradation. The absence of a gene encoding a functional extradiol dioxygenase will typically result in the concomitant loss of the ability to degrade toluene in organisms that break down chlorobenzene via a chlorocatechol *ortho*-cleavage pathway, since methylaromatics can be degraded by *ortho*-cleavage pathways only in extraordinary cases (Rojo *et al.*, 1987). Here, the recruitment of a chlorocatechol *meta*-cleavage pathway has equipped strain GJ31 with the capability to degrade both chlorobenzene and toluene (Mars *et al.*, 1997), despite a non-functional extradiol dioxygenase in the upper pathway gene cluster.

Various features of the *cbzTEXGS* cluster of strain GJ31 deserve special attention. The coding and non-coding regions are nearly identical to the clusters of other chlorobenzene-degrading organisms using a *meta*-cleavage pathway (Göbel *et al.*, 2004), and contain only an incomplete *meta*-cleavage pathway. Significant induction of CbzE in response to chlorobenzene was observed, but

also a high level of transcripts even in fructose-grown cells, which may prevent accumulation of toxic catechols (Schweigert *et al.*, 2001). CbzE is part of the subfamily I.2.C of extradiol dioxygenases (Eltis & Bolin, 1996). Various genes encoding enzymes of this subfamily have been recently identified, including in the course of genome sequencing projects (e.g. *Leptothrix cholodnii* SP-6, NC_010524; *Acidovorax* sp. JS42, NC_008782; *Verminephrobacter eiseniae* EF01-2, NC_008786; *C. necator* JMP134, NC_007348). From such accumulated knowledge, gene clusters comprising genes encoding subfamily I.2.C extradiol dioxygenases typically encode enzymes of both the hydrolytic and the oxalocrotonate branch of the *meta*-cleavage pathway. Only part of the conserved structure is retained in the form of the *cbzTEXG* genes, and a GST-encoding gene has not been observed to be encoded in other clusters that contain genes encoding subfamily I.2.C extradiol dioxygenases. These clusters are typically preceded by genes encoding either an aniline dioxygenase (Fukumori & Saint, 2001; Urata *et al.*, 2004) or a multicomponent phenol hydroxylase (Zhu *et al.*, 2008). Due to the presence of 177 bases in front of the ferredoxin gene homologous to genes encoding the NADH oxidoreductase of multicomponent phenol hydroxylase, it can be suggested that the *cbz* cluster was recruited from such a source.

Although downstream of the *cbzE* cluster *cbzJ* encodes a functional and novel type of 2-hydroxypent-2,4-dienoate hydratase, the origin of which remains to be established, the *cbzE* cluster in concert with the upper pathway gene cluster is not sufficient to allow mineralization of chlorobenzene via 2-hydroxymuconate as an intermediate (Fig. 4). Two additional catabolic gene clusters, which could partially (*mhp* gene cluster) or completely (*nah* gene cluster) fulfil the role of creating a complete pathway, could be identified here. However, the transcription of both gene clusters was only slightly elevated in response to chlorobenzene. Nevertheless, the significant level of transcription of the *nah* gene cluster even in the absence of chlorobenzene supports the notion that it is responsible for completion of the metabolic route.

The chlorobenzene degradative pathway in strain GJ31 (Fig. 4) thus seems to be a mosaic constructed of (i) a chromosomal toluene pathway, (ii) a phenol metabolic cluster for the recruitment of *cbzE*, (iii) a phenylpropionate pathway, and (iv) an incomplete NAH pathway. Strain GJ31 is an example of an organism obtained from enrichment cultures able to use a chloroaromatic compound as the growth substrate in which the degradative pathway is the product of patchwork assembly.

ACKNOWLEDGEMENTS

This work was financed by a grant from the Deutsche Forschungsgemeinschaft (Re659/7) and by the European Union, contract EVK1-CT1999-00023 'MAROC'. We thank Professor Timothy D. H. Bugg, University of Warwick, for a sample of 2,3-dihydroxyphenylpropionate and Melissa Wos-Oxley for critical reading of the manuscript.

REFERENCES

Barnes, M. R., Duetz, W. A. & Williams, P. A. (1997). A 3-(3-hydroxyphenyl)propionic acid catabolic pathway in *Rhodococcus globerulus* PWD1: cloning and characterization of the *hpp* operon. *J Bacteriol* **179**, 6145–6153.

Bartels, F., Backhaus, S., Moore, E. R. B., Timmis, K. N. & Hofer, B. (1999). Occurrence and expression of glutathione-*S*-transferase-encoding *bphK* genes in *Burkholderia* sp. strain LB400 and other biphenyl-utilizing bacteria. *Microbiology* **145**, 2821–2834.

Beil, S., Happe, B., Timmis, K. N. & Pieper, D. H. (1997). Genetic and biochemical characterization of the broad-spectrum chlorobenzene dioxygenase from *Burkholderia* sp. strain PS12: dechlorination of 1,2,4,5-tetrachlorobenzene. *Eur J Biochem* **247**, 190–199.

Beil, S., Mason, J. R., Timmis, K. N. & Pieper, D. H. (1998). Identification of chlorobenzene dioxygenase sequence elements involved in dechlorination of 1,2,4,5-tetrachlorobenzene. *J Bacteriol* **180**, 5520–5528.

Beil, S., Timmis, K. N. & Pieper, D. H. (1999). Genetic and biochemical analyses of the *tec* operon suggest a route for evolution of chlorobenzene degradation genes. *J Bacteriol* **181**, 341–346.

Bosch, R., Garcia-Valdes, E. & Moore, E. R. B. (2000). Complete nucleotide sequence and evolutionary significance of a chromosomally encoded naphthalene-degradation lower pathway from *Pseudomonas stutzeri* AN10. *Gene* **245**, 65–74.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**, 248–254.

Bramucci, M., Chen, M. & Nagarajan, V. (2006). Genetic organization of a plasmid from an industrial wastewater bioreactor. *Appl Microbiol Biotechnol* **71**, 67–74.

Bugg, T. D. H. (1993). Overproduction, purification and properties of 2,3-dihydroxyphenylpropionate 1,2-dioxygenase from *Escherichia coli. Biochim Biophys Acta* **1202**, 258–264.

Collinsworth, W. L., Chapman, P. J. & Dagley, S. (1973). Stereospecific enzymes in the degradation of aromatic compounds by *Pseudomonas putida. J Bacteriol* **113**, 922–931.

Diaz, E., Ferrandez, A., Prieto, M. A. & Garcia, J. L. (2001). Biodegradation of aromatic compounds by *Escherichia coli. Microbiol Mol Biol Rev* **65**, 523–569.

Dorn, E. & Knackmuss, H.-J. (1978a). Chemical structure and biodegradability of halogenated aromatic compounds. Two catechol 1,2-dioxygenases from a 3-chlorobenzoate-grown pseudomonad. *Biochem J* **174**, 73–84.

Dorn, E. & Knackmuss, H.-J. (1978b). Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of catechol. *Biochem J* **174**, 85–94.

Eltis, L. D. & Bolin, J. T. (1996). Evolutionary relationships among extradiol dioxygenases. *J Bacteriol* **178**, 5930–5937.

Ferrandez, A., Garcia, J. L. & Diaz, E. (1997). Genetic characterization and expression in heterologous hosts of the 3-(3-hydroxyphenyl)-propionate catabolic pathway of *Escherichia coli* K-12. *J Bacteriol* **179**, 2573–2581.

Friemann, R., Lee, K., Brown, E. N., Gibson, D. T., Eklund, H. & Ramaswamy, S. (2009). Structures of the multicomponent Rieske non-heme iron toluene 2,3-dioxygenase enzyme system. *Acta Crystallogr D Biol Crystallogr* **65**, 24–33.

Fukumori, F. & Saint, C. P. (2001). Complete nucleotide sequence of the catechol metabolic region of plasmid pTDN1. *J Gen Appl Microbiol* **47**, 329–333.

Gibson, D. T. & Parales, R. E. (2000). Aromatic hydrocarbon dioxygenases in environmental biotechnology. *Curr Opin Biotechnol* **11**, 236–243.

Gibson, D. T., Koch, J. R., Schuld, C. L. & Kallio, R. E. (1968). Oxidative degradation of aromatic hydrocarbons by microorganisms. II. Metabolism of halogenated aromatic hydrocarbons. *Biochemistry* **7**, 3795–3802.

Göbel, M., Kranz, O. H., Kaschabek, S. R., Schmidt, E., Pieper, D. H. & Reineke, W. (2004). Microorganisms degrading chlorobenzene via a *meta*-cleavage pathway harbor highly similar chlorocatechol 2,3-dioxygenase-encoding gene clusters. *Arch Microbiol* **182**, 147–156.

Gross, R., Guzman, C. A., Sebaihia, M., dos Santos, V. A., Pieper, D. H., Koebnik, R., Lechner, M., Bartels, D., Buhrmester, J. & other authors (2008). The missing link: *Bordetella petrii* is endowed with both the metabolic versatility of environmental bacteria and virulence traits of pathogenic Bordetellae. *BMC Genomics* **9**, 449.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.

Herrmann, H., Lauf, U. & Müller, C. (1998). The transposable elements resident on the plasmids of *Pseudomonas putida* strain H, Tn*5501* and Tn*5502*, are cryptic transposons of the Tn*3* family. *Mol Gen Genet* **259**, 674–678.

Hülsmeyer, M., Hecht, H. J., Niefind, K., Hofer, B., Eltis, L. D., Timmis, K. N. & Schomburg, D. (1998). Crystal structure of *cis*-biphenyl-2,3-dihydrodiol-2,3-dehydrogenase from a PCB degrader at 2.0 Å resolution. *Protein Sci* **7**, 1286–1293.

Junca, H. & Pieper, D. H. (2004). Functional gene diversity analysis in BTEX contaminated soils by means of PCR-SSCP DNA fingerprinting: comparative diversity assessment against bacterial isolates and PCR-DNA clone libraries. *Environ Microbiol* **6**, 95–110.

Kaschabek, S. R. & Reineke, W. (1992). Maleylacetate reductase of *Pseudomonas* sp. strain B13: dechlorination of chloromaleylacetates, metabolites in the degradation of chloroaromatic compounds. *Arch Microbiol* **158**, 412–417.

Kaschabek, S. R., Kasberg, T., Müller, D., Mars, A. E., Janssen, D. B. & Reineke, W. (1998). Degradation of chloroaromatics: purification and characterization of a novel type of chlorocatechol 2,3-dioxygenase of *Pseudomonas putida* GJ31. *J Bacteriol* **180**, 296–302.

Lau, P. C. K., Bergeron, H., Labbe, D., Wang, Y., Brousseau, R. & Gibson, D. T. (1994). Sequence and expression of the *todGIH* genes involved in the last three steps of toluene degradation by *Pseudomonas putida* F1. *Gene* **146**, 7–13.

Lauf, U., Müller, C. & Herrmann, H. (1998). The transposable elements resident on the plasmids of *Pseudomonas putida* strain H, Tn*5501* and Tn*5502*, are cryptic transposons of the Tn*3* family. *Mol Gen Genet* **259**, 674–678.

Mars, A. E., Kasberg, T., Kaschabek, S. R., van Agteren, M. H., Janssen, D. B. & Reineke, W. (1997). Microbial degradation of chloroaromatics: use of the *meta*-cleavage pathway for mineralization of chlorobenzene. *J Bacteriol* **179**, 4530–4537.

Mars, A. E., Kingma, J., Kaschabek, S. R., Reineke, W. & Janssen, D. B. (1999). Conversion of 3-chlorocatechol by various catechol 2,3-dioxygenases and sequence analysis of the chlorocatechol dioxygenase region of *Pseudomonas putida* GJ31. *J Bacteriol* **181**, 1309–1318.

Menn, F.-M., Zylstra, G. J. & Gibson, D. T. (1991). Location and sequence of the *todF* gene encoding 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase in *Pseudomonas putida*. *Gene* **104**, 91–94.

Mosqueda, G., Ramos-Gonzalez, M. I. & Ramos, J. L. (1999). Toluene metabolism by the solvent-tolerant *Pseudomonas putida* DOT-T1 strain, and its role in solvent impermeabilization. *Gene* **232**, 69–76.

Müller, T. A., Werlen, C., Spain, J. & van der Meer, J. R. (2003). Evolution of a chlorobenzene degradative pathway among bacteria in a contaminated groundwater mediated by a genomic island in *Ralstonia*. *Environ Microbiol* **5**, 163–173.

Nozaki, M. (1970). Metapyrocatechase (*Pseudomonas*). *Methods Enzymol* **17A**, 522–525.

Oldenhuis, R., Kuijk, L., Lammers, A., Janssen, D. B. & Witholt, B. (1989). Degradation of chlorinated and non-chlorinated aromatic solvents in soil suspension by pure bacterial cultures. *Appl Microbiol Biotechnol* **30**, 211–217.

Olivera, E. R., Minambres, B., Garcia, B., Muniz, C., Moreno, M. A., Ferrandez, A., Diaz, E., Garcia, J. L. & Luengo, J. M. (1998). Molecular characterization of the phenylacetic acid catabolic pathway in *Pseudomonas putida* U: the phenylacetyl-CoA catabolon. *Proc Natl Acad Sci U S A* **95**, 6419–6424.

Peters, M., Heinaru, E., Talpsep, E., Wand, H., Stottmeister, U., Heinaru, A. & Nurk, A. (1997). Acquisition of a deliberately introduced phenol degradation operon, *pheBA*, by different indigenous *Pseudomonas* species. *Appl Environ Microbiol* **63**, 4899–4906.

Poelarends, G. J., Veetil, V. P. & Whitman, C. P. (2008). The chemical versatility of the β-α-β fold: catalytic promiscuity and divergent evolution in the tautomerase superfamily. *Cell Mol Life Sci* **65**, 3606–3618.

Pohlman, R. F., Genetti, H. D. & Winans, S. C. (1994). Entry exclusion of the IncN plasmid pKM101 is mediated by a single hydrophilic protein containing a lipid attachment motif. *Plasmid* **31**, 158–165.

Powell, J. A. C. & Archer, J. A. C. (1998). Molecular characterisation of a *Rhodococcus ohp* operon. *Antonie Van Leeuwenhoek* **74**, 175–188.

Providenti, M. A., Shaye, R. E., Lynes, K. D., McKenna, N. T., O'Brien, J. M., Rosolen, S., Wyndham, R. C. & Lambert, I. B. (2006). The locus coding for the 3-nitrobenzoate dioxygenase of *Comamonas* sp. strain JS46 is flanked by IS*1071* elements and is subject to deletion and inversion events. *Appl Environ Microbiol* **72**, 2651–2660.

Ravatn, R., Studer, S., Springael, D., Zehnder, A. J. B. & van der Meer, J. R. (1998). Chromosomal integration, tandem amplification, and deamplification in *Pseudomonas putida* F1 of a 105-kilobase genetic element containing the chlorocatechol degradative genes from *Pseudomonas* sp. strain B13. *J Bacteriol* **180**, 4360–4369.

Reineke, W. (1998). Development of hybrid strains for the mineralization of chloroaromatics by patchwork assembly. *Annu Rev Microbiol* **52**, 287–331.

Reineke, W. (2001). Aerobic and anaerobic biodegradation potentials of microorganisms. In *Biodegradation and Persistence (The Handbook of Environmental Chemistry, vol. 2K)*, pp. 1–161. Edited by B. Beek. Berlin/Heidelberg: Springer Verlag.

Rojo, F., Pieper, D. H., Engesser, K.-H., Knackmuss, H.-J. & Timmis, K. N. (1987). Assemblage of ortho cleavage route for simultaneous degradation of chloro- and methylaromatics. *Science* **238**, 1395–1398.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schmidt, E. & Knackmuss, H.-J. (1980). Chemical structure and biodegradability of halogenated aromatic compounds. Conversion of chlorinated muconic acids into maleoylacetic acid. *Biochem J* **192**, 339–347.

Schmidt, E., Remberg, G. & Knackmuss, H.-J. (1980). Chemical structure and biodegradability of halogenated aromatic compounds. Halogenated muconic acids as intermediates. *Biochem J* **192**, 331–337.

Schwarzenbach, R. P., Gschwend, P. M. & Imboden, D. M. (2003). *Environmental Organic Chemistry*, 2nd edn. Hoboken, NJ: Wiley-Interscience.

Schweigert, N., Zehnder, A. J. B. & Eggen, R. I. L. (2001). Chemical properties of catechols and their molecular modes of toxic action in cells, from microorganisms to mammals. *Environ Microbiol* **3**, 81–91.

Sherratt, D. (1989). Tn*3* and related transposable elements: site-specific recombination and transposition. In *Mobile DNA*, pp. 1302–1308. Edited by D. E. Berg & M. M. Howe. Washington, DC: American Society for Microbiology.

Shingler, V., Powlowski, J. & Marklund, U. (1992). Nucleotide sequence and functional analysis of the complete phenol/3,4-dimethylphenol catabolic pathway of *Pseudomonas* sp. strain CF600. *J Bacteriol* **174**, 711–724.

Sota, M., Yano, H., Nagata, Y., Ohtsubo, Y., Genka, H., Anbutsu, H., Kawasaki, H. & Tsuda, M. (2006). Functional analysis of unique class II insertion sequence IS*1071*. *Appl Environ Microbiol* **72**, 291–297.

Stanley, T. M., Johnson, W. H., Burks, E. A., Whitman, C. P., Hwang, C. C. & Cook, P. F. (2000). Expression and stereochemical and isotope effect studies of active 4-oxalocrotonate decarboxylase. *Biochemistry* **39**, 3514.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Tropel, D., Meyer, C., Armengaud, J. & Jouanneau, Y. (2002). Ferredoxin-mediated reactivation of the chlorocatechol 2,3-dioxygenase from *Pseudomonas putida* GJ31. *Arch Microbiol* **177**, 345–351.

Urata, M., Uchida, E., Nojiri, H., Omori, T., Obo, R., Miyaura, N. & Ouchiyama, N. (2004). Genes involved in aniline degradation by *Delftia acidovorans* strain 7N and its distribution in the natural environment. *Biosci Biotechnol Biochem* **68**, 2457–2465.

van der Meer, J. R., Zehnder, A. J. B. & de Vos, W. M. (1991). Identification of a novel composite transposable element, Tn*5280*, carrying chlorobenzene dioxygenase genes of *Pseudomonas* sp. strain P51. *J Bacteriol* **173**, 7077–7083.

Volff, J. N., Eichenseer, C., Viell, P., Piendl, W. & Altenbuchner, J. (1996). Nucleotide sequence and role in DNA amplification of the direct repeats composing the amplificable element AUD1 of *Streptomyces lividans* 66. *Mol Microbiol* **21**, 1037–1047.

Wackett, L. P. & Hershberger, C. D. (2001). Biocatalysis and biodegradation. *Microbial Transformation of Organic Compounds*. Washington, DC: American Society for Microbiology.

Wehmhöner, D., Häussler, S., Tümmler, B., Jänsch, L., Bredenbruch, F., Wehland, J. & Steinmetz, I. (2003). Inter- and intraclonal diversity of the *Pseudomonas aeruginosa* proteome manifests within the secretome. *J Bacteriol* **185**, 5807–5814.

Wheatcroft, R. & Williams, P. A. (1981). Rapid methods for the study of both stable and unstable plasmids in *Pseudomonas*. *J Gen Microbiol* **124**, 433–437.

Witzig, R., Junca, H., Hecht, H. J. & Pieper, D. H. (2006). Assessment of toluene/biphenyl dioxygenase gene diversity in benzene-polluted soils: links between benzene biodegradation and genes similar to those encoding isopropylbenzene dioxygenases. *Appl Environ Microbiol* **72**, 3504–3514.

Witzig, R., Aly, H. A., Strömpl, C., Wray, V., Junca, H. & Pieper, D. H. (2007). Molecular detection and diversity of novel diterpenoid dioxygenase DitA1 genes from proteobacterial strains and soil samples. *Environ Microbiol* **9**, 1202–1218.

Yano, H., Garruto, C. E., Sota, M., Ohtsubo, Y., Nagata, Y., Zylstra, G. J., Williams, P. A. & Tsuda, M. (2007). Complete sequence determination combined with analysis of transposition/site-specific recombination events to explain genetic organization of IncP-7 TOL plasmid pWW53 and related mobile genetic elements. *J Mol Biol* **369**, 11–26.

Zhu, C., Zhang, L. & Zhao, L. (2008). Molecular cloning, genetic organization of gene cluster encoding phenol hydroxylase and catechol 2,3-dioxygenase in *Alcaligenes faecalis* IS-46. *World J Microbiol Biotechnol* **24**, 1687–1695.

Zylstra, G. J. & Gibson, D. T. (1989). Toluene degradation by *Pseudomonas putida* F1. Nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli*. *J Biol Chem* **264**, 14940–14946.

Edited by: H. L. Drake