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ORIGINAL PAPER

Grouping of phenol hydroxylase and catechol 2,3-dioxygenase genes among phenol- and *p*-cresol-degrading *Pseudomonas* species and biotypes

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Abstract Phenol- and *p*-cresol-degrading pseudomonads isolated from phenol-polluted water were analysed by the sequences of a large subunit of multicomponent phenol hydroxylase (LmPH) and catechol 2,3-dioxygenase (C23O), as well as according to the structure of the plasmid-borne *pheBA* operon encoding catechol 1, 2-dioxygenase and single component phenol hydoxylase. Comparison of the *carA* gene sequences (encodes the small subunit of carbamoylphosphate synthase) between the strains showed species- and biotype-specific phylogenetic grouping. LmPHs and C23Os clustered similarly in *P. fluorescens* biotype B, whereas in *P. mendocina* strains strong genetic heterogeneity became evident. *P. fluorescens* strains from biotypes C and F were shown to possess the *pheBA* operon, which was also detected in the majority of *P. putida* biotype B strains which use the *ortho* pathway for phenol degradation. Six strains forming a separate LmPH cluster were described as the first pseudomonads possessing the Mop type LmPHs. Two strains of this cluster possessed the genes for both single and multicomponent PHs, and two had genetic rearrangements in the *pheBA* operon leading to the deletion of the *pheA* gene. Our data suggest that few central routes for the degradation of phenolic compounds may emerge in bacteria as a result of the combination of genetically diverse catabolic genes.

Keywords Phenol and *p*-cresol-degrading bacteria · Carbamoylphosphate synthase gene (*carA*) · Phenol hydroxylase · Catechol 2,3-dioxygenase · *pheBA* operon

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Introduction

The metabolic capacity of indigenous microbial consortia present in a continuously polluted environment may reflect adaptation of various catabolic pathways in pollutant-degrading indigenous bacterial strains (El Fantroussi and Agathos 2005). Numerous studies on microbial catabolism of phenol and alkylphenols (e.g. cresols) have led to a deeper understanding of the degradation of aromatic compounds, in terms of enzymology, genetics, microbial diversity, and bioremediation. The microbial strategy for aerobic degradation of aromatic compounds involves two critical steps: first, the ring hydroxylation of adjacent carbon atoms and second, the ring cleavage of the resulting catecholic intermediates. In the case of phenol degradation, the aromatic ring is first monohydroxylated by phenol hydroxylase (PH, phenol 2-monooxygenase, EC 1.14.13.7) at *ortho* position to the pre-existing hydroxyl group. The next step is catalysed by either catechol 1,2-dioxygenase (C12O, initiating the *ortho* pathway leading to formation of succinyl-CoA and acetyl-CoA) or catechol 2,3-dioxygenase (C23O, initiating the *meta* pathway leading to formation of pyruvate and acetaldehyde) (Nozaki et al. 1970; Mason and Cammack 1992).

Two different types of PHs have been identified: single (sPH) and multicomponent (mPH) (Shingler et al. 1989; Kukor and Olsen 1990; Nurk et al. 1991). PHs from *Pseudomonas pickettii* PKO1 (encoded by *tbuD*) and *Pseudomonas* sp. EST1001 (encoded by *pheA*) belong to the first group. The *tbuD* gene is co-transcribed with the

C23O gene (*tbuE*) and the latter gene shares the operon with the *pheB* gene encoding C12O (Kukor and Olsen 1991; Kivisaar et al. 1991). mPHs are composed of six subunits and have a very similar protein structure; the catabolic site is located within the largest subunit (Watanabe et al. 1998). The genetic organisation of operons coding for mPHs is different: the genes that code for the *dmp* type (from *Pseudomonas* CF600) and the *mop* type (from *Acinetobacter calcoaceticus* NCIB8250) of mPHs are located upstream of the genes that are followed by the genes that code for C23O and C12O, respectively (Shingler et al. 1992; Ehrt et al. 1995). This may reflect the preference of different mPHs to catabolise differently substituted phenols.

C23O (catechol:oxygen 2,3-oxidoreductase; EC 1.3.11.2) is a key enzyme of many bacterial pathways for the degradation of aromatic compounds. The majority of C23Os are phylogenetically closely related, belonging to the subfamily 1.2.A of the 1.2 extradiol dioxygenase family, and are of particular importance in the degradation of monocyclic aromatic compounds (Eltis and Bolin 1996). Methyl-substituted aromatic compounds are usually degraded through the *meta* pathway catalysed by C23O or via o*rtho* ring cleavage of protocatechuate (*ortho*^{prot}) by using *p*-cresol methylhydroxylase as a key enzyme of this route (Dagley and Patel 1957).

We have previously shown that phenol/*p*-cresol-degraders isolated from river water continuously polluted with phenolic compounds of oil shale ash leachate group into three different catabolic types: (a) phenol and *p*-cresol are both degraded via the catechol *meta* pathway (*meta–meta* type), (b) phenol is degraded via the catechol *ortho* and *p*-cresol, via the protocatechuate *ortho* pathway (*ortho–ortho*^{prot}), or (c) phenol is degraded via the catechol *meta* and *p*-cresol via the protocatechuate *ortho* pathway (*meta–ortho*^{prot}) (Heinaru et al. 2000). The main aim of the present study was to compare the gene sequences of PHs and C23Os, responsible for the efficient degradation of phenols in these strains, in order to elucidate their species- and biotype-specific clustering, and to prove the assumption that different combinations of genetically diverse catabolic genes may lead to functionally similar central routes of degradation of phenolic compounds.

Materials and methods

Bacterial strains and growth conditions

A total of 38 phenol- and *p*-cresol-degrading strains from the genus *Pseudomonas* isolated from phenol-polluted river water were studied (Table 1). The strains and the culture media have been described in our previous work (Heinaru et al. 2000).

DNA extraction, plasmid characterisation and Southern hybridisation

Genomic DNA of bacterial strains was extracted from 2 ml of Luria-Bertani (LB) overnight culture using the UltraClean microbial DNA isolation kit (MO BIO Laboratories), according to the manufacturer's instructions. The extracted DNA was stored at −20°C. Plasmid DNA was detected according to the procedures of Connors and Barnsley (1982). To specify the location of phenol degradation genes in studied strains, a Southern blot analysis of plasmid DNA was performed as described by Sambrook et al. (1989). Digestion of DNA with a restriction enzyme was performed according to the manufacturer's (Fermentas) guidelines. For Southern hybridisation of the isolated plasmid DNA, *pheA* probes were generated via PCR from the strain *Pseudomonas putida* EST1412 using *pheA* primers (Table 2) as previously described (Heinaru et al. 2001). LmPH gene probes for hybridisation were generated from the strains PC16, PC17, PC30 and P69 using primers described in Table 2. The purified DNA fragments were labelled with [α^{32}P]dCTP (Amersham Pharmacia Biotech, Inc.) using a DNA labelling kit (DecaLabel™, Fermentas). Autoradiograms were analysed using PhosphorImager (Molecular Dynamics).

Primers and PCR conditions

The extracted DNA was quantified spectrophotometrically and subjected to PCR amplification. The primers used for the amplification of the studied genes are listed in Table 2. Amplification was performed by using a 25 µl mixture containing about 15 ng of template DNA, 1 × PCR buffer consisting of $(NH_4)_2SO_4$ as provided in the Fermentas *Taq* DNA polymerase kit, 200 µM concentration of each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 20 pmol of each primer, 0.5 U of *Taq* DNA polymerase (Fermentas). The amplification and sequence determination of 16S rRNA genes of the strains were performed as described in our previous work (Heinaru et al. 2001). Amplification of the *carA* gene used as a species- and biotype-specific marker in pseudomonads was performed as described by Hilario et al. (2004).

The PCR programs for the amplification of 580 bp LmPH gene fragments, as well as for the 924 bp C23O and the 947 bp *pheA* gene fragments have been described previously (Futamata et al. 2001; Junca and Pieper 2003; Heinaru et al. 2005). Step cycles of the

Table 1 Bacterial strains used and their GenBank accession numbers for *carA* and 16S rDNA

Strain	Identified by *carA*	Strain abbreviation	Accession no. for *carA*	Accession no. for 16S rDNA
PC1	*P. mendocina*	Pmen PC1	DQ178181	AF232713
PC2	*P. mendocina*	Pmen PC2	DQ178182	DQ178219
PC3	*P. mendocina*	Pmen PC3	DQ178183	ND
PC4	*P. mendocina*	Pmen PC4	DQ178184	DQ178220
PC5	*P. mendocina*	Pmen PC5	DQ178185	DQ178221
PC6	*P. mendocina*	Pmen PC6	DQ178186	DQ178222
PC7	*P. mendocina*	Pmen PC7	DQ178187	DQ178223
PC8	*P. mendocina*	Pmen PC8	DQ178188	ND
PC9	*P. mendocina*	Pmen PC9	DQ178189	ND
PC10	*P. mendocina*	Pmen PC10	DQ178190	DQ178224
PC11	*P. mendocina*	Pmen PC11	DQ178191	ND
PC12	*P. mendocina*	Pmen PC12	DQ178192	DQ178225
PC13	*P. putida* B	Ppu PC13	DQ178193	ND
PC14	*P. putida* B	Ppu PC14	DQ178194	AY973266
PC15	*P. putida* B	Ppu PC15	DQ178195	AY973267
PC16	*P. putida* B	Ppu PC16	DQ178196	AY918067
PC17	*P. fluorescens* F	PfF PC17	DQ178197	AY538263
PC18	*P. fluorescens* B	PfB PC18	DQ178198	AF228366
PC19	*P. mendocina*	Pmen PC19	DQ178199	DQ178226
PC20	*P. fluorescens* F	PfF PC20	DQ178200	AY538264
PC21	*P. fluorescens* B	PfB PC21	DQ178201	DQ178227
PC22	*P. fluorescens* B	PfB PC22	DQ178202	DQ178228
PC23	*P. fluorescens* B	PfB PC23	DQ178203	DQ178229
PC24	*P. fluorescens* C	PfC PC24	DQ178204	AF228367
PC25	*P. fluorescens* C	PfC PC25	DQ178205	ND
PC26	*P. fluorescens* C	PfC PC26	DQ178206	ND
PC28	*P. fluorescens* C	PfC PC28	DQ178207	ND
PC30	*P. putida* B	Ppu PC30	DQ178208	AY918068
PC31	*P. fluorescens* C	PfC PC31	DQ178209	ND
PC32	*P. fluorescens* B	PfB PC32	DQ178210	DQ178230
PC33	*P. fluorescens* B	PfB PC33	DQ178211	DQ178231
PC34	*P. fluorescens* B	PfB PC34	DQ178212	DQ178232
PC35	*P. putida* B	Ppu PC35	DQ178213	ND
PC36	*P. putida* B	Ppu PC36	DQ178214	DQ178233
PC37	*P. fluorescens* B	PfB PC37	DQ178215	DQ178234
PC38	*P. fluorescens* B	PfB PC38	DQ178216	DQ178235
PC39	*P. putida* B	Ppu PC39	DQ178217	ND
P69	*P. fluorescens* F	PfF P69	DQ178218	AY973265

ND not determined

probe reactions for the *pheBA* operon were as follows: the *pheB* gene fragment (847 bp) and IS*1472* (330 bp) were amplified at 94°C for 1 min, 52°C for 45 s, 7°C for 1 min; IS*1411* (996 bp) and IRL (1112 bp) were amplified at 94°C for 1 min, 61°C for 45 s, 72°C for 1 min. All amplifications were performed for 32 cycles, and an additional extension step of 10 min at 72°C was employed in the end. To evaluate PCR product sizes, 5 µl of the PCR reaction was analysed using agarose gel electrophoresis (1.5–2.0%, 1× TAE running buffer, at 100 V). DNA bands were visualised by ethidium bromide (final concentration, 0.5 µg l^{-1}) staining.

PCR products were purified from 2% agarose gels using the QIAquick Gel Extraction Kit (Qiagen) or UltraClean™ 15 DNA Purification Kit (MO BIO Laboratories) according to the instructions provided. The PCR products were ligated into pTZ57R using the InsT/Aclone™ PCR Product Cloning Kit (MBI Fermentas) and cloned into *E. coli* DH5α competent cells (Inoue et al. 1990). The *E. coli* cells were cultured on LB medium at 37°C, containing ampicillin 15 µg ml^{-1}, isopropyl β-D-thiogalactopyranoside 48 µg ml^{-1} and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside 80 µg ml^{-1}. For the detection of LmPH and C23O genes, *Pseudomonas* sp. CF600 was used as a reference strain (Shingler et al. 1989).

Analysis of the *pheBA* operon

The genetic organisation of the *pheBA* operon was determined by PCR using the primer pairs shown in

Table 2 PCR primers used for amplification of DNA probes

Probe/Primer	Nucleotide sequence (5′→3′)	References
LmPH/pheUf	CCAGGSBGARAARGAGARGAARCT	Futamata et al. (2001)
pheMHr	GATBGGCACRTTGTCTTC	
pheA/pheA1	CAGGATCGAATATCGGTGGCCTCG	Heinaru et al. (2000)
pheA2	CTTCACGCTGGCGTAACCAATCGC	
pheB/pheB1	TCGTTGCTGGTCTCGACC	This study
pheB2	TTGCAGCTCCTCTTCTGC	
IS*1472*/IS1	GTTGTTCCTGATCGAGATG	Peters et al. (2004)
IS2	GCCGCGGTCGCCCAGATAGC	
IS*1411*/pheA3	GCGATTGGTTACGCCAGCGTGAAG	Peters et al. (2004)
IS3	GCTTGAGGCAGGGCTTCTTGCG	This study
IRL/pheA1	CAGGATCGAATATCGGTGGCCTCG	Heinaru et al. (2000)
IRL	CGGTCAGATTTTTTCTACACCC	This study
C23O/ORF-F	AGGTGWCGTSATGAAMAAAGG	Junca and Pieper (2003)
ORF-R	TYAGGTSAKMACGTTCAKGAA	
carA/carA-F	TTCAACACCGCCATGACCGG	Hilario et al. (2004)
carA-R	TGATGRCCSAGGCAGATRCC	

Table 2. The sequences of amplified products were compared with those of the *pheBA* operon in the GenBank. The cells for enzyme assay were harvested in the late exponential growth phase. Crude extracts were prepared and enzyme activity was assayed as described previously (Heinaru et al. 2000). The activity of sPH was monitored spectrophotometrically by examining oxidation of NADPH at 340 nm (Hegeman 1966). Protein concentration was measured according to the Bradford method (1976). Enzyme activities were determined from at least three separate independent experiments. *P. putida* EST1412 was used as a reference strain for the measurement of the sPH activity (Kivisaar et al. 1990).

Northern hybridisation

For northern analysis, bacterial strains were grown overnight in a minimal medium containing 0.2% (w/v) casamino acids (CAA) and inoculated into fresh CAA medium supplemented with 2.5 mM phenol as inducer at initial optical density of ca. 0.1 at 580 nm. Total RNA was extracted from exponential-phase cultures using the Nucleospin® RNA II extraction kit (Macherey-Nagel) according to the manufacturers' instructions. About 20 μg of total RNA per lane was used for northern blot analysis. Agarose-formaldehyde gel electrophoresis was performed as described in Sambrook et al. (1989). The RNA was transferred onto nylon membrane (Hybond-N$^+$; Amersham Bioscience) using the capillary method and fixed by UV cross-linking. Gene-specific DNA probes were generated by PCR (Table 2) and hybridisation was performed according to standard protocols (Sambrook et al. 1989).

DNA sequencing and phylogenetic analyses

Nucleotide sequencing of the cloned inserts was carried out on an ABI Prism™ 377 DNA sequencer (Perkin-Elmer) using the DYEnamic™ ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) and the protocols provided by the manufacturer. The GenBank database search was conducted using BLAST programs. CLUSTAL W version 1.83 was used for the sequence alignments (Thompson et al. 1994). Phylogenetic trees were constructed from the evolutionary distance matrix by the neighbour-joining method (Saitaou and Nei 1987). The sequence data reported in this study has been deposited in GenBank under accession numbers AY875721–AY875748, and DQ387868 for LmPH, and AY887949–AY887972 for C23O genes. The *carA* and 16S rRNA gene sequences obtained in this study are available in GenBank under the accession numbers shown in Table 1.

Results and discussion

Species- and biotype-specific grouping of pseudomonads based on sequences of the *carA* gene

Although sequencing of 16S rRNA genes has proven to be a valuable tool in taxonomic analysis of bacteria (Woese 1987), its application in the case of the genus *Pseudomonas* is limited, especially within the *fluorescens* lineage of pseudomonads (Hilario et al. 2004). Quite expectedly, we were unable to construct a phylogenetic tree precisely distinguishing the 27 *Pseudomonas* strains studied in this work (Table 1) according to the determined 16S rRNA gene sequences. The high identity

level of this gene within *Pseudomonas* strains, especially among *P. fluorescens* biotypes (about 99%) hampers its use in phylogenetic analysis. Therefore, other genes including the *carA* gene encoding the small subunit of carbamoylphosphate synthase (Lawson et al. 1996) have been used for more precise phylogenetic analysis (Eisen 1995; Yamamoto et al. 2000). Carbamoylphosphate synthase genes are essentially immune to horizontal gene transfer (Cammarano et al. 2002) and evolve much faster than ribosomal rRNA genes, thus providing higher resolution power to discriminate between closely related species (Yamamoto et al. 2000). In this study, the phylogenetic tree of 38 strains of phenol/*p*-cresol-degraders and the reference strains was constructed on the basis of the partial sequences (617 bp) of the *carA* gene. As expected, it revealed distinct groupings of the *Pseudomonas fluorescens* biotypes B, C and F (Fig. 1, groups I, IV and IIIb, respectively), *P. mendocina* (Fig. 1, group II) and *P. putida* species (Fig. 1, group IIIa). It also became obvious that *P. putida* biotype B is closely related to *P. fluorescens* biotype F, groups IIIa and IIIb, respectively. The latter result is also supported by the fact that no metabolic differences between these strains were detected by Hilario et al. (2004).

Phylogeny of LmPHs and C23Os

In order to study diversity between the catabolic genes, the partial sequences of LmPH (580 bp) and C23O (924 bp) genes of the phenol/*p*-cresol-degraders were analysed. The respective PCR amplifications resulted in 29 LmPH and 24 C23O positive strains. The deduced amino acid sequences of these PCR products were aligned against sequences of reference strains. The phylogenetic tree of the proteins deduced from the sequences

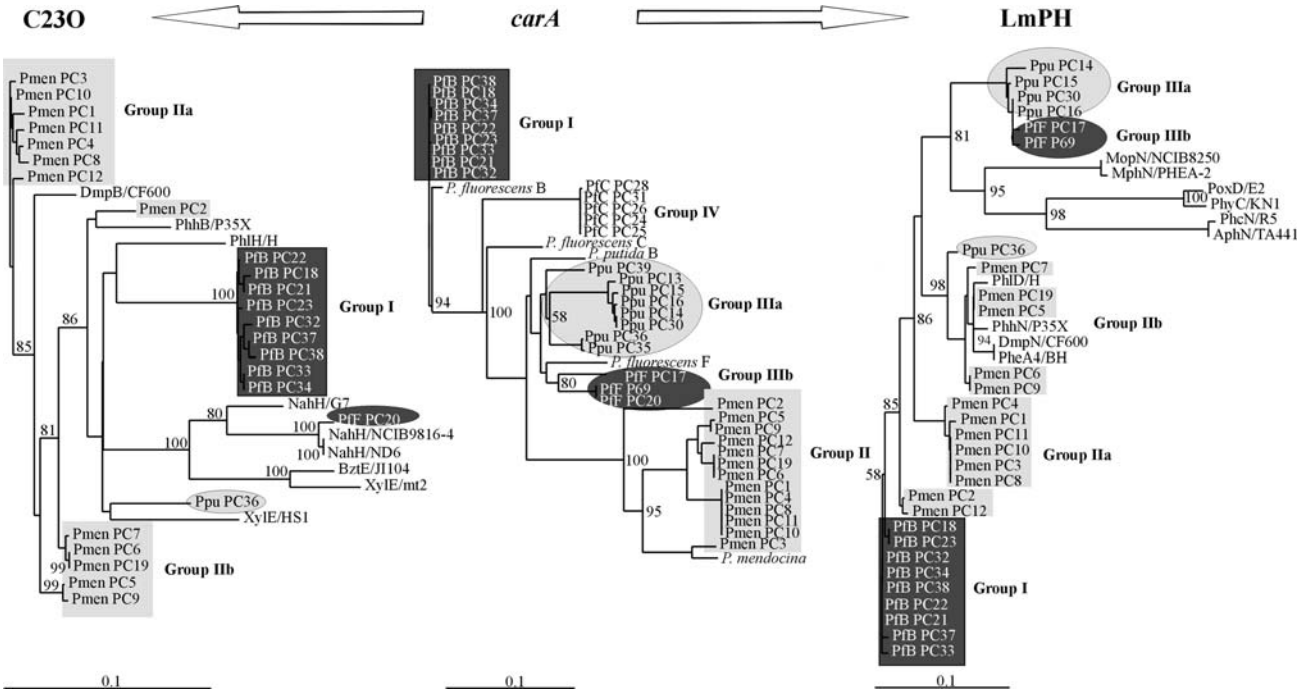

Fig. 1 Neighbour-joining trees based on the 617 bp of *carA* gene sequences, on the deduced amino acid sequences of the LmPHs (580 bp) and C23Os (924 bp) of the phenol/*p*-cresol degraders and reference strains. The *carA* gene sequences obtained from the GenBank database belong to the following type strains: *P. mendocina* ICMP 13540 (AJ414222), *P. putida* biotype B ICMP 13630 (AJ414224), *P. fluorescens* biotype B ICMP 13619 (AJ414216), biotype C ICMP (AJ414217) and biotype F ICMP 13616 (AJ414218). The sequences obtained in this study were aligned with the known LmPHs: MopN from *Acinetobacter calcoaceticus* NCIB8250 (Z36909), MphN from *A. calcoaceticus* PHEA-2 (AJ564846), PoxD from *Ralstonia* sp. E2 (AF026065), PhyC from *Ralstonia* sp. KN1 (AB031996), PhcN from *Comamonas testosteroni* R5 (AB024741), AphN from *C. testsseroni* TA441 (AB006479), PhlD from *P. putida* H (X80765), PhhN from *P. putida* P35X (X79063), DmpN from *Pseudomonas* sp. CF600 (M60276), PheA4 from *P. putida* BH (D28864), and C23Os: DmpB from *P. putida* CF600 (M33263), PhlH from *P. putida* H (X80765), NahH *P. putida* G7 (P08127), NahH from *P. putida* NCIB9816-4 (AA064305), NahH from *Pseudomonas* sp. ND6 (NP-943120), BztE from *P. aeruginosa* JI104 (X60740), XylE from *P. putida* mt2 (V01161), XylE from *P. putida* HS1 (M65205), PhhB from *P. putida* P35X (X77856). Bootstrap values (per 1000 trials) higher than 50% are indicated at the nodes. The scale bars represent 0.1 substitutions per nucleotide (*carA*) or amino acid site (LmPH, C23O). The groups of different species and biotypes revealed according to the *carA* gene sequence analysis are designated using different grades of *grey* shading and shapes, which are used to track the position of these strains in phylogenetic trees of C23O and LmPH

revealed four main groups (I, IIa, IIb, IIIa + IIIb) of LmPH genes (Fig. 1). Only in the case of two strains, Pmen PC2 and Pmen PC12, the LmPH genes grouped outside those four clusters. Six strains (Ppu PC14-PC16, Ppu PC30, PfF PC17, PfF P69) harbouring group IIIa + IIIb LmPHs did not contain C23O genes. The phylogenetic tree for the C23O genes indicated the presence of three distinct groups (I, IIa, IIb) with the exception of strains Pmen PC2, Ppu PC36 and PfF PC20. Notably, the LmPH gene was absent in the PfF PC20 strain.

Comparison of the clustering data **(**Fig. 1**)** of ten strains belonging to the *meta–ortho*^{prot} degradation type of phenol and *p*-cresol (Table 3) shows that all nine *P. fluorescens* biotype B strains analysed (PC18, PC21-PC23, PC32-PC34, PC37, PC38) form a unique set within the group I LmPH and the group I C23O genes. We suppose that it may reflect selective pressure of phenolic pollutants in the environment on *P. fluorescens* biotype B bacteria. Pmen PC12 strain from the *meta–ortho*^{prot} type is exceptional, because it clusters according to the LmPH analysis with *P. mendocina* strain PC2, while according to C23O analysis, with the group IIa (Fig. 1; Table 3).

P. mendocina strains (12 out of 13) degrade phenol and *p*-cresol through the *meta* pathway. The deduced amino acid sequences of LmPH and C23O are not congruent with *carA* (Fig. 1). Two main clusters of strains were revealed: six strains (PC1, PC3, PC4, PC8, PC10, PC11) form group IIa and five strains (PC5-PC7, PC9, PC19) form group IIb, according to sequences of LmPH and C23O. LmPHs from group IIb are closely related to those of the reference strains and belong to the Dmp family (DmpN, PhlD, PhhN and PheA4) (Fig. 1). The C23O genes of group IIb are similar to DmpB from *Pseudomonas* sp. CF600. LmPH gene from the strain Pmen PC2 groups together with that of the strain Pmen PC12, and C23Os of Pmen PC2 and PhhB are similar (Table 3). The *P. putida* strain PC36 from biotype B possessing the *meta–meta* catabolic type for degradation of phenol and *p*-cresol is exceptional: it is the only strain in this phylogenetic group with the C23O gene (Table 3). LmPH of the strain Ppu PC36 groups close to LmPHs of the Dmp family, and C23O close to *xylE* of *P. putida* HS1 (Fig. 1). Thus, our analysis concerning catabolic genes showed genetic heterogeneity of *P. mendocina* strains that may reflect adaptation of these bacteria to presence of phenolic pollutants in the environment.

Strains harbouring sPH

The *pheBA* operon determines the synthesis of sPH (encoded by the *pheA*) and C12O (encoded by the *pheB*), it is plasmid-borne and there is strong evidence of horizontal transfer of this operon in nature (Peters et al. 1997). The *pheBA* operon is flanked by two IS elements, IS*1472* and IS*1411*. The promoter of the *pheBA* operon is located upstream of IS*1472* (Kasak et al. 1993). IS*1411* was discovered as a consequence of insertional activation of the promoterless *pheBA* genes in *P. putida* due to the presence of outward-directed promoters at the left end of IS*1411* (Kallastu et al. 1998). In this study we analysed the presence of the

Table 3 Grouping of phenol hydroxylase and catechol 2,3-dioxygenase genes among catabolic types and *Pseudomonas* species

Species and biotypes identified by *carA*	Strain designation (PC)	Catabolic type of phenol–*p*-cresol degradation	LmPH group[a]	C23O group[a]	*pheBA* operon[a,b]
P. fluorescens B	18, 21 –23, 32–34, 37, 38	*meta–ortho*^{prot}	I	I	–
P. mendocina	1, 3, 4, 8, 10, 11	*meta–meta*	IIa	IIa	–
	5–7, 9, 19	*meta–meta*	IIb	IIb	–
	2	*meta–meta*	Similar to 12	Similar to PhhB	–
	12	*meta–ortho*^{prot}	Similar to 2	Similar to IIa	–
P. putida B	14, 15	*ortho–ortho*^{prot}	IIIa	–	–
	16	*ortho–ortho*^{prot}	IIIa	–	+
	30	*ortho–ortho*^{prot}	IIIa	–	d
	13, 35, 39	*ortho–ortho*^{prot}	–	–	+
	36	*meta–meta*	IIb	Similar to XylE	–
P. fluorescens F	17	*ortho–ortho*^{prot}	IIIb	–	d
	P69	*ortho*^{c}	IIIb	–	+
	20	*ortho–meta*	–	Similar to NahH	+
P. fluorescens C	24–26, 28, 31	*ortho–ortho*^{prot}	–	–	+

ortho^{c}, the strain does not degrade *p*-cresol; *d, pheA* gene from *pheBA* operon absent

[a] –, *pheBA* operon, genes for LmPH or C23O are absent

[b] +, *pheBA* operon is present

pheBA operon in our strains by using PCR analysis with the *pheBA*-specific primers described in Table 2 (data not shown). We found this operon in 13 strains belonging to the *ortho–ortho*^{prot} type of degradation of phenol and *p*-cresol (Table 3). However, these strains belong to different *Pseudomonas* species and biotypes: *P. fluorescens* biotype C (PC strains 24–26, 28, 31), *P. putida* biotype B (PC strains 13, 16, 35, 39, 30) and *P. fluorescens* biotype F (PC strains 20, 17 and P69) (Table 3). The strain PfF PC20 is particularly interesting, as it is the only strain with the *ortho–meta* type of degradation for phenol and *p*-cresol possessing the C23O gene related to NahH gene of *P. putida* strain NCIB 9816-4. Therefore, it is reasonable to assume that the *pheBA* operon was transferred horizontally into this strain.

Co-presence of mPH and sPH genes

LmPH group IIIa + IIIb is the most distant cluster revealed by phylogenetic analyses. It contains four strains of *P. putida* B (PC14-16, PC30) and two strains

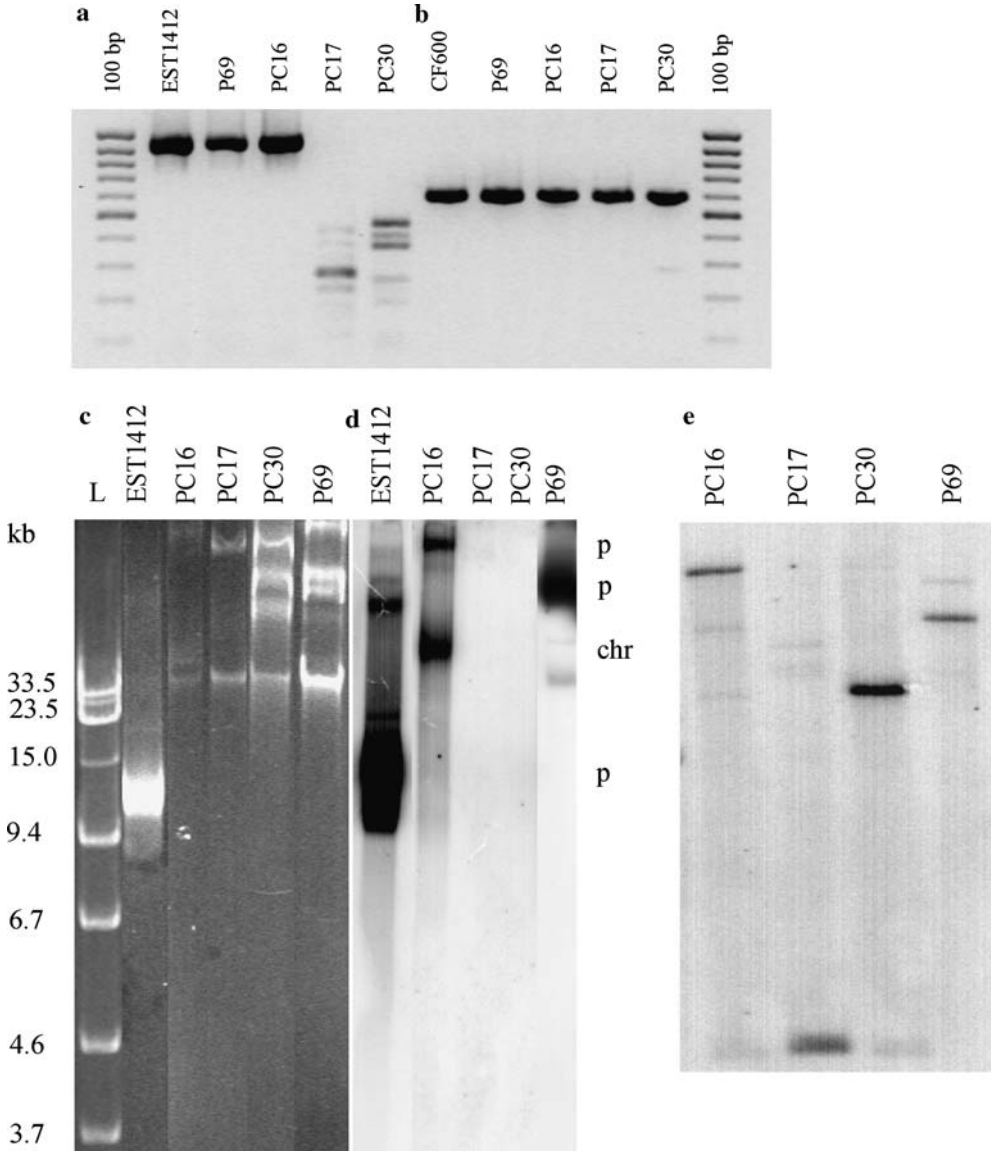

Fig. 2 Agarose gel electrophoresis of *pheA* (**a**) and LmPH (**b**) gene fragments amplified from the *pheA*$^{+}$ reference strain *P. putida* EST1412, the LmPH^{+} strain *Pseudomonas* sp. CF600 and the strains P69, PC16, PC17 and PC30. Plasmids retrieved from the *pheBA* operon possessing strains P69, PC16, PC17, PC30 and the positive control EST1412 (**c**) and Southern hybridisation of plasmids with the *pheA* gene probe (**d**). L, Bsp681/XhoI restriction fragments of λDNA were used as the DNA size standards; *chr* chromosomal DNA; *p* plasmid DNA. Autoradiograph of Southern analysis of total EcoRI-digested DNA of the studied strains using the LmPH gene probe (**e**). The strain designations are indicated above the *lanes*

of *P. fluorescens* F (PC17 and P69). Figure 1 indicates that MopN and MphN genes of reference strains are the nearest neighbours of group IIIa + IIIb. These six strains are the first examples of pseudomonads possessing the Mop type LmPHs coupled with the C12O gene. As shown in the previous paragraph, the *pheBA* operon was detected in four strains out of them: Ppu PC16, Ppu PC30, PfF PC17, PfF P69 (Table 3). The amplified products of sPHs and LmPHs from these strains were analysed together with those from reference strains using agarose gel electrophoresis. The co-presence of the two different genes of phenol hydroxylases, sPH (encoded by *pheA*) and mPH was found in strains Ppu PC16 and PfF P69**,** but not in PfF PC17 and Ppu PC30 (Fig. 2a, b). To examine the localisation of the gene coding for PHs, the plasmids of the four strains and the reference strain EST1412 were separated by electrophoresis and analysed by Southern hybridisation (Fig. 2c, d). We found that a *pheA* gene-specific probe yielded a strong hybridisation signal with the plasmid DNA of the strains Ppu PC16 and PfF P69, whereas no signal was detected in strains PfF PC17 and Ppu PC30. Applying an LmPH gene-specific probe, no hybridisation was detected in the case of the plasmid DNA from these strains, whereas the digested genomic DNA (EcoRI) showed positive hybridisation signal in all four lanes (Fig. 2e). These results verify the presence of the *pheA* gene in plasmids of two strains and indicate that the LmPH gene is chromosomally encoded in all four strains.

We analysed the structure of the *pheBA* operon of these four strains and in PfC PC24 by sequencing the amplified PCR products generated by PCR primers shown in Table 2. We revealed genetic changes in the *pheBA* operon in some strains compared to the original *pheBA* operon of pAT1140 (Kasak et al. 1993). The promoterless *pheBA* operon of pEST1412 was used as a control for the detection of the outward-directed promoter on the left side of IS*1411* (Kallastu et al. 1998). According to the analysis, the *pheBA* operon from strains PfC PC24 and PfF P69 is similar to that of pAT1140, whereas in Ppu PC16 it lacks IS*1411* (Fig. 3). The strains PfF PC17 and Ppu PC30 carry an incomplete *pheBA* operon without the *pheA*. It is known that some bacteria employ more than one pathway to degrade hydrocarbons, which allows formation of novel mixed metabolic pathways and may explain why bacterial strains capable of growing on contaminants emerge so quickly (Notomista et al. 2003). DNA

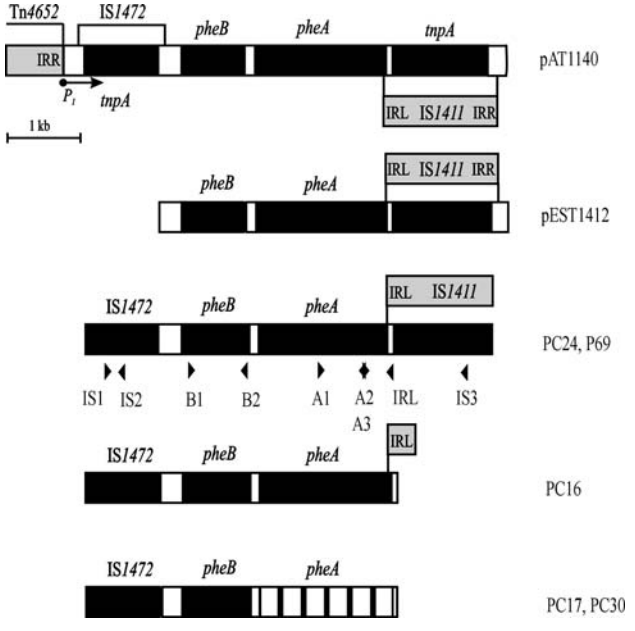

Fig. 3 Schematic organisation of the *pheBA* operon in strains possessing different level of the expression of sPH. Kasak and Kallastu determined the original structure of the pheBA operon in pAT1140 with co-workers (Kasak et al. 1993; Kallastu et al. 1998) (GenBank accession No. M57500). The *black* boxes show the locations of the *pheBA* genes and the transposase genes of IS*1472* and IS*1411*. The *open* boxes represent the intergenic regions, the *fragmented pheA* box indicates the eliminated region, and the *grey* box indicates the presence of the left end of inverted repeats (IRL) of IS*1411*

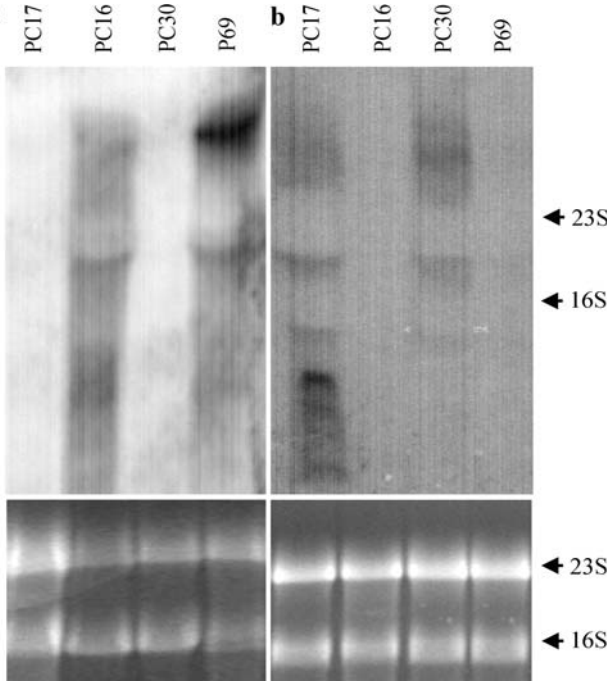

Fig. 4 Northern hybridisation analysis of *pheA* (**a**) and LmPH genes (**b**) from strains PC16, PC17, PC30 and P69. Hybridisation signals (*top*) and ethidium-bromide-stained total RNA in denatured agarose gel (*bottom*) is shown as a loading control

Table 4 PMO activity in *pheA*-expressing and -nonexpressing strains

Strain	Type of PH gene[a]	sPH[b] (nmol min^{-1} mg^{-1})
P. putida EST1412	*pheA*	1,008 ± 9
P. fluorescens C PC24	*pheA*	536 ± 73
P. fluorescens F PC17	mPH –	< 1
P. putida B PC30	mPH –	< 4
P. putida B PC16	mPH *pheA*	50 ± 6
P. fluorescens F P69	mPH *pheA*	469 ± 50
Pseudomonas sp. CF600	mPH	< 1

[a] mPH—contains a multicomponent phenol hydroxylase gene; *pheA*—contains a single component phenol hydroxylase gene;–, the *pheA* gene absent

[b] Specific activity values of sPH are means of three independent experiments ± standard errors of the means

fragments can move into new hosts to be incorporated or recombined, creating new mosaic genetic structures (van der Meer and Sentchilo 2003).

To evaluate the expression of the *pheBA* operon and the LmPH gene in the strains Ppu PC16, Ppu PC30, PfF PC17, PfF P69, a northern blot analysis and enzymatic study of sPH was performed. A hybridisation signal was detected with the *pheA* gene probe in case of RNA samples isolated from phenol-induced cells of Ppu PC16 and PfF P69 but not in PC17 and PC30, as expected from the absence of *pheA* in those strains (Fig. 4a). Interestingly, using a LmPH gene probe, RNAs from strains PC17 and PC30 hybridised, but no hybridisation signal was detected with RNA from Ppu PC16 and PfF P69 (Fig. 4b), although the respective gene was present in the genome of these strains (Fig. 2e). Specific activities of sPH of different *pheBA* positive strains and of the reference strain *P. putida* EST1412 showed that no sPH activity was present in cell extracts of PfF PC17 and Ppu PC30, and only low activity was detected in Ppu PC16. In contrast, the strains PfC PC24 and PfF P69 had a high sPH activity (Table 4). It has been previously reported that the mPH activity cannot be measured in crude cell extracts of pseudomonads (Powlowski and Shingler 1990). Consequently, the activity revealed in our assay should belong to sPH. This is further supported by the fact that mPH activity was not detectable using this assay in the reference strain *Pseudomonas* sp. CF600 that uses mPH for the growth on phenol (Table 4).

Thus, our results indicate elimination of the gene coding for sPH from the *pheBA* operon in strains PfF PC17 and Ppu PC30. However, these strains are still able to grow on phenol because they contain the LmPH gene. We assume that these strains have acquired the full-length *pheBA* operon through horizontal gene transfer, and further genetic rearrangements have led to the loss of the *pheA* gene. In strains Ppu PC16 and PfF P69 possessing genes for both sPH and mPH, the plasmid-encoded *pheA* gene is functional and expresses sPH activity but LmPH gene is probably not expressed as not detected in northern analysis. Next, we will attempt to find out how the LmPH gene has been inactivated in these strains and why some of the studied strains have selected sPH and others mPH for phenol degradation. It may be that the ability of bacterial cells to choose between different catabolic operons with similar function can be an effective and a flexible strategy to survive and function in a natural consortium under the conditions of heavy phenolic pollution.

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