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Three types of phenol and *p*-cresol catabolism in phenol- and *p*-cresol-degrading bacteria isolated from river water continuously polluted with phenolic compounds

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Abstract

A total of 39 phenol- and *p*-cresol-degraders isolated from the river water continuously polluted with phenolic compounds of oil shale leachate were studied. Species identification by BIOLOG GN analysis revealed 21 strains of *Pseudomonas fluorescens* (4, 8 and 9 of biotypes A, C and G, respectively), 12 of *Pseudomonas mendocina*, four of *Pseudomonas putida* biotype A1, one of *Pseudomonas corrugata* and one of *Acinetobacter genospecies* 15. Computer-assisted analysis of rep-PCR fingerprints clustered the strains into groups with good concordance with the BIOLOG GN data. Three main catabolic types of degradation of phenol and *p*-cresol were revealed. Type I, or *meta-meta* type (15 strains), was characterized by *meta* cleavage of catechol by catechol 2,3-dioxygenase (C23O) during the growth on phenol and *p*-cresol. These strains carried C23O genes which gave PCR products with specific *xyl*E-gene primers. Type II, or *ortho-ortho* type (13 strains), was characterized by the degradation of phenol through *ortho* fission of catechol by catechol 1,2-dioxygenase (C12O) and *p*-cresol via *ortho* cleavage of protocatechuic acid by protocatechuate 3,4-dioxygenase (PC34O). These strains carried phenol monooxygenase gene which gave PCR products with *phe*A-gene primers. Type III, or *meta-ortho* type (11 strains), was characterized by the degradation of phenol by C23O and *p*-cresol via the protocatechuate *ortho* pathway by the induction of PC34O and this carried C23O genes which gave PCR products with C23O-gene primers, but not with specific *xyl*E-gene primers. In type III strains phenol also induced the *p*-cresol protocatechuate pathway, as revealed by the induction of *p*-cresol methylhydroxylase. These results demonstrate multiplicity of catabolic types of degradation of phenol and *p*-cresol and the existence of characteristic assemblages of species and specific genotypes among the strains isolated from the polluted river water.

Keywords: Phenol-degrader; *p*-Cresol-degrader; Catechol 1,2-dioxygenase; Catechol 2,3-dioxygenase; Protocatechuate 3,4-dioxygenase; *xyl*E; *phe*B; PCR fingerprint; *Pseudomonas*

1. Introduction

Phenetic and genetic analysis of bacterial populations in environmental samples is becoming increasingly important in microbial ecology research, especially because of their potential role in biorestoration of polluted sites [1]. Important progress in the elucidation of individual metabolic pathways has been gained, but little is known of the pathways operating in natural communities, where extensive sharing of nutritional resources is common [2].

Studies of major pathways for catabolism of aromatic compounds in bacteria have revealed that, while different enzymes carry out initial conversion steps, compounds are transformed into a limited number of central intermediates, such as protocatechuate and (substituted) catechols. These dihydroxylated intermediates are channeled into the *ortho* cleavage pathway (also termed β-ketoadipate pathway) or the *meta* cleavage pathway [3,4]. *ortho* Ring cleavage consists of two parallel branches for the catabolism of catechol and protocatechuate in *Pseudomonas putida* [5]. Catechol and protocatechuate ring fission steps are catalyzed by catechol 1,2-dioxygenase (C12O; encoded by *cat*A) and protocatechuate 3,4-dioxygenase (PC34O; encoded by *pca*HG), respectively [6]. Methyl-substituted aromatic substrates are generally degraded via *meta* fission catalyzed by catechol 2,3-dioxygenases (C23O), whereas the ring fission of nonsubstituted aromatics and chloroaromatics occurs through *ortho* cleavage [7,8]. Substrate

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mixtures will induce both pathways (if they exist) and, therefore, if their substrate specificity allows attack on different compounds in the mixture, substrates may be channelled into pathways that could result in the accumulation of dead end metabolites. Two distinct catabolic routes have been described for *p*-cresol, one being initiated by the oxidation of the methyl group of *p*-cresol to a corresponding carboxyl group [9]. The first step in this route is catalyzed by *p*-cresol methylhydroxylase (PCMH; EC 1.17.99.1) [10]. Catabolism of the intermediate *p*-hydroxybenzoate (POB) to protocatechuate is catalyzed by *p*-hydroxybenzoate hydroxylase (POBH) encoded by a *pob*A gene [6]. In *P. putida* the genes encoding the *p*-cresol metabolism via the protocatechuate *ortho* cleavage pathway can be carried on plasmid [11]. In the other route, the methyl group of *p*-cresol remains intact and 4-methylcatechol is formed, which is further catabolized via the *meta* pathway of catechol [12].

Phenol hydroxylase (EC 1.14.13.7) catalyzes the initial step of microbial phenol degradation by the insertion of oxygen into the aromatic ring, providing catechol. In the archetypal phenol- and *p*-cresol-degrader *P. putida* strain U, phenol- and *p*-cresol catabolism is carried out by chromosomally encoded *meta* cleavage enzymes [13]. *Pseudomonas* sp. strain CF600 grows efficiently on phenol, cresols and 3,4-dimethylphenol [14,15]. The PHE plasmids pVI150 of the strain *Pseudomonas* sp. C600 [14] and pPGH1 of *P. putida* H [16,17] encode multicomponent phenol hydroxylase and C23O, allowing degradation of phenol and *p*-cresol via *meta* cleavage of catechol [18]. In *Pseudomonas pickettii* PKO1 the utilization of phenol and *p*-cresol is determined by phenol/cresol hydroxylase, a product of *tbu*D gene, which occurs near the genes for the *meta* cleavage pathway [19,20]. Phenol degradation by the *ortho* pathway is specified by plasmids in *Pseudomonas* sp. strain EST1001 [21]. The *phe*BA operon in the plasmid pEST1026 encodes a single phenol monooxygenase (PMO) and C12O [22,23].

Six years after deliberate release of bacteria carrying the phenol degradation operon, *phe*BA, to a river continuously polluted with phenolic compounds of the oil shale industry leachate, we have found the acquisition of the operon by indigenous microbes [24]. The principal aromatic pollutants in the leachate of an oil shale ash mound (formed as a by-product of Estonian oil shale processing and impacted with chemical industry process waters) are phenol and *p*-cresol [25]. Indigenous microorganisms are usually more competitive in using different mixtures [4]. The aim of the present study was to determine whether phenol- and *p*-cresol-degrading bacteria isolated from the river water continuously polluted with phenolic compounds have one predominant or, alternatively, diverse catabolic types of degradation of phenol and *p*-cresol. The assemblages of species, specific genotypes (i.e. *xyl* or *phe*) and induction of key enzymes were also studied to characterize the catabolic types.

2. Materials and methods

2.1. Site description and sample collection

Bacteria were isolated from river water, polluted by the phenolic oil-shale ash leachate. Samples were taken from a site in Northeast Estonia on 14 September and 30 November 1993, 2 March, 2 June and 15 September 1994 and 18 April 1995. The wastewater discharges downstream through the Channel (sites 8, 7 and 6), Kohtla River (sites 5 and 1) and the Purtse River (sites 3 and 2) to the Baltic Sea, at a distance of 22.1 km between the dump area and the Baltic Sea (Fig. 1). Water samples were collected from a depth of 15 cm in sterile 1000-ml bottles, transported on ice to the laboratory and analyzed on the same day.

2.2. Bacterial strains and culture conditions

Thirty nine phenol- and *p*-cresol-degrading (PHE^+*p*-CRE^+) strains (named as PC1 to PC39) were isolated on agar plates incubated at 22°C for 5 days. The minimal media containing M9 salts [26] and trace elements [27] were supplemented with phenol (2.5 mM), benzoate, *m*-toluate, salicylate (all 5 mM) or *n*-heptane (in vapor phase). The Luria-Bertani (LB) and LB+carbenicillin (1000 μg ml^{-1}) agar plates were also performed. Pure cultures were stored in 20% glycerol at −70°C.

Liquid cultures were grown in minimal medium supplemented with either phenol (2.5 mM) or *p*-cresol (1.3 mM) at 30°C on a rotary shaker (180 rpm). Bacterial growth was measured spectrophotometrically at 580 nm. Induction experiments were performed in 250-ml Erlenmeyer flasks containing 50 ml of minimal medium supplemented with 0.2% (w/v) Casamino acids (CAA) and an inducing carbon source: phenol (2.5 mM), *p*-cresol (1.3 mM), benzoate (5 mM) or POB (2.5 mM). For the induction fresh medium was inoculated with 10 ml of late exponential

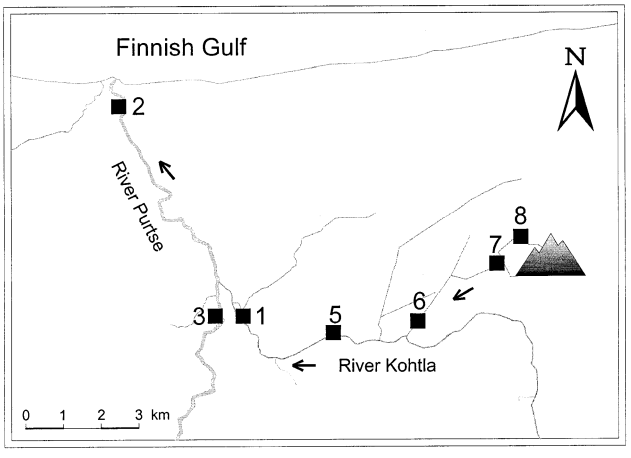

Fig. 1. Map of the sample collection sites in northeast Estonia. The black rectangles and numbers indicate the sampling sites, the arrows show the route of phenolic leachate from the ash dump area (site 8) to the Finnish Gulf (site 2).

phase culture grown on the same medium but without the inducer, and the induction of catabolic enzymes was studied in crude extracts from the late exponential phase cells. Noninduced enzyme levels were determined in extracts from cells sampled before addition of the inducer. Reference strains for the measurement of the activities of C23O and PMO, and *xyl*E and *phe*A genes were the phenol-utilizing strain *P. putida* E2 with a large 83-kb plasmid pWWO^{mut90} that carries the TOL plasmid pWWO *meta* cleavage operon [28] and *P. putida* EST1026 (pEST1026) [29], respectively.

2.3. Identification of strains

The isolates were Gram stained by the classical staining method and the 3% (w/v) KOH test was also performed. Standard methods [30] were used to characterize the isolates: fluorescence on King's B medium; growth at 42°C; denitrification (production of N_2 and/or N_2O and growth); hydrolysis of gelatin and casein, and production of levan in sucrose-rich medium (5.0% sucrose and 2.3% Bacto Nutrient Agar, Difco). The purity of cultures was tested by plating on LB agar and microscopic observation. Single colonies of each isolate (on LB agar plates) were picked and streaked onto BUGM (Biolog universal growth medium; Biolog, Hayward, CA, USA) agar plates. The identification of strains was carried out according to the BIOLOG GN identification manual. Using the MicroLog™ 2 software, the identification of microbes was acknowledged when the similarity index was 0.5 or more.

2.4. Genomic fingerprints

Colony rep-PCR fingerprinting patterns from bacterial genomic DNA were generated with BOXA1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′) [31]. Each isolate was grown on a phenol minimal agar plate for 24–48 h, and a small amount of cells from a colony was picked by a needle and suspended in 25 μl of MilliQ H_2O. The cells were lysed by heating at 96°C for 10 min, immediately cooled on ice, centrifuged, and 25 μl of PCR mixture was added. The PCR was performed in 50 μl (total volume) reaction mixture containing lysed cells, 30 pmol of BOXA1R primer, 50 mM KCl, 20 mM Tris-HCl (pH 9.0), 2.5 mM $MgCl_2$, deoxynucleoside triphosphates (Pharmacia Biotech) each at the concentration of 200 μM and 1 u of *Taq* polymerase (Pharmacia Biotech). The reactions were performed in a DNA thermal cycler 'UNO Thermoblock', Biometra (1 cycle at 95°C for 6 min followed by 30 cycles at 94°C for 1 min, 53°C for 1 min, 65°C for 8 min, 68°C for 2 min and a final extension at 65°C for 15 min prior to cold storage at 4°C). Samples of the rep-PCR products were separated by electrophoresis on horizontal 1.5% (w/v) agarose gels. Post-electrophoresis staining of gels was done in 0.5 μg ml^{-1} of ethidium bromide. Computer-assisted analysis of rep-PCR genomic fingerprints with GelCompar software (version 4.0; Applied Maths, Kortrijk, Belgium) was performed. Gel images were scanned (HP ScanJet II), stored on disk as TIFF files, converted and normalized using 1-kb DNA ladder (GeneRuler™, Fermentas) as a molecular size marker. The 'rolling disk' background subtraction method was applied. To analyze rep-PCR patterns, similarity matrices of whole densitometric curves of the gel tracks were calculated using the pair-wise Pearson's product-moment correlation coefficient. Cluster analysis of similarity matrices was performed using Ward's method [32].

2.5. DNA techniques

Plasmid DNA was detected by the procedures of Connors and Barnsley [33]. Plasmid profiles were visualized by ethidium bromide staining of 0.8% (w/v) agarose gels. PCR amplification was used to detect the presence of *xyl*E [28] and *phe*A [29] genes in the native bacterial strains. The *xyl*E primer pairs E1 (5′-TCAAGGTTGTGGATGAGGATGC-3′) and E2 (5′-AGAACACTTCGTTGCGGTTACC-3′) were designed according to the DNA sequence of the conserved region of the C23O gene of *xyl*E and should result in a PCR amplification product of 594 bp [28]. The primer pairs E2 (see above) and E3 (5′-GGTATGGCGGCTGTGCGTTTCGACCA-3′), designed by Dr. Allan Nurk from our laboratory for the detection of C23O genes, were expected to yield the PCR amplification product of 356 bp. The PCR primers *phe*A1 (5′-CAGGATCGAATATCGGTGGCCTCG-3′) and *phe*A2 (5′-CTTCACGCTGGCGTAACCAATCGC-3′) were used for the detection of the *phe*A gene [25]. Oligonucleotide primers were synthesized by Biometra (Gottingen, Germany). The PCR conditions employed have been previously described [28]. The amplified products were analyzed by electrophoresis on 0.8% (w/v) agarose gels stained with ethidium bromide. Colony hybridization has been described previously [28].

2.6. Enzyme activity assay

Crude extracts were prepared from cells washed twice with cold K_2HPO_4-KH_2PO_4 buffer (100 mM; pH 7.5), resuspended in the same buffer and sonically disrupted. Unbroken cells and cell debris were removed by centrifugation at 12000×*g* for 30 min at 4°C. PMO and POBH activities were determined spectrophotometrically in 50 mM phosphate buffer (pH 7.5) by monitoring the disappearance of NADPH at 340 nm as described in [34] and [35], respectively. For the measurement of endogenous oxidation of NADPH, the substrate (phenol or POB) was omitted from the reaction mixture. The assay mixture for POBH contained 0.3 mM NADPH and 0.01 mM FAD as cofactors. The reaction was started by the addition of POB at the final concentration of 0.4 mM. Activities of C12O and C23O were measured spectrophotomet-

rically by monitoring the formation of reaction products at 260 and 375 nm, respectively [36,37]. The crude extracts used for C12O activity measurements were pre-treated for 5 min with H_2O_2 (0.01%), that suppresses the activity of C23O [38]. PC34O activity was measured by determining the decrease of absorption at 290 nm due to the oxidation of protocatechuate [39]. PCMH activity was assayed by the procedure of Kim et al. [40]. The cell extracts (10 μg of total protein) were subjected to polyacrylamide gel electrophoresis (PAGE) under nondenaturating conditions. The acrylamide concentrations were 4.5% (w/v) and 7.5% (w/v) in the stacking and resolving gels, respectively. The gel was stained for PCMH activity in the following reaction mixture: 100 mM phosphate buffer (pH 7.5); *p*-cresol, 1.3 mM; Nitro Blue tetrazolium, 0.2 mg ml^{-1}; phenazine methosulfate, 0.2 mg ml^{-1}. Protein concentrations were measured by the method of Bradford [41] with bovine serum albumin as a standard.

3. Results

3.1. Identification and characterization of phenol- and p-cresol-degrading (PHE^+p-CRE^+) strains

Thirty nine PHE^+p-CRE^+ bacteria were isolated from seven different sampling sites, at six different times, from six different selective media and from LB-agar plates (Table 1). Most PHE^+p-CRE^+ isolates belonged to the genus *Pseudomonas*. Species identification by BIOLOG GN analysis revealed 21 strains of *Pseudomonas fluorescens* (4, 8 and 9 of biotypes A, C and G, respectively), 12 of *Pseudomonas mendocina*, four of *P. putida* biotype A1, one of *Pseudomonas corrugata* and one strain was *Acinetobacter genospecies* 15 (Table 1). Additional characterization of pseudomonads by classical microbiological tests showed that *P. putida* and *P. fluorescens* strains were producing fluorescent pigment on King's B medium, *P. mendocina* strains (10 out of 12) were growing at 42°C, *P. fluorescens* biotype C strains were able to hydrolyze gelatin and casein, *P. fluorescens* strains (except three of them) and PC3 were denitrifiers, and only three strains, PC17, PC20 and PC24, produced levan from sucrose. Hence, these tests were in good agreement with characterization of the genus - *Pseudomonas* by Palleroni [30], except for proteolytic activity, which was characteristic for *P. fluorescens* biotype C strains only.

3.2. Heterogeneity of rep-PCR genomic fingerprints in PHE^+p-CRE^+ new isolates

In order to verify heterogeneity among the PHE^+p-CRE^+ bacteria, all strains we tested by rep-PCR chromosomal fingerprinting using a BOXAIR primer and computer-assisted analysis that indicated concordance with the species and *P. fluorescens* biotype identification results

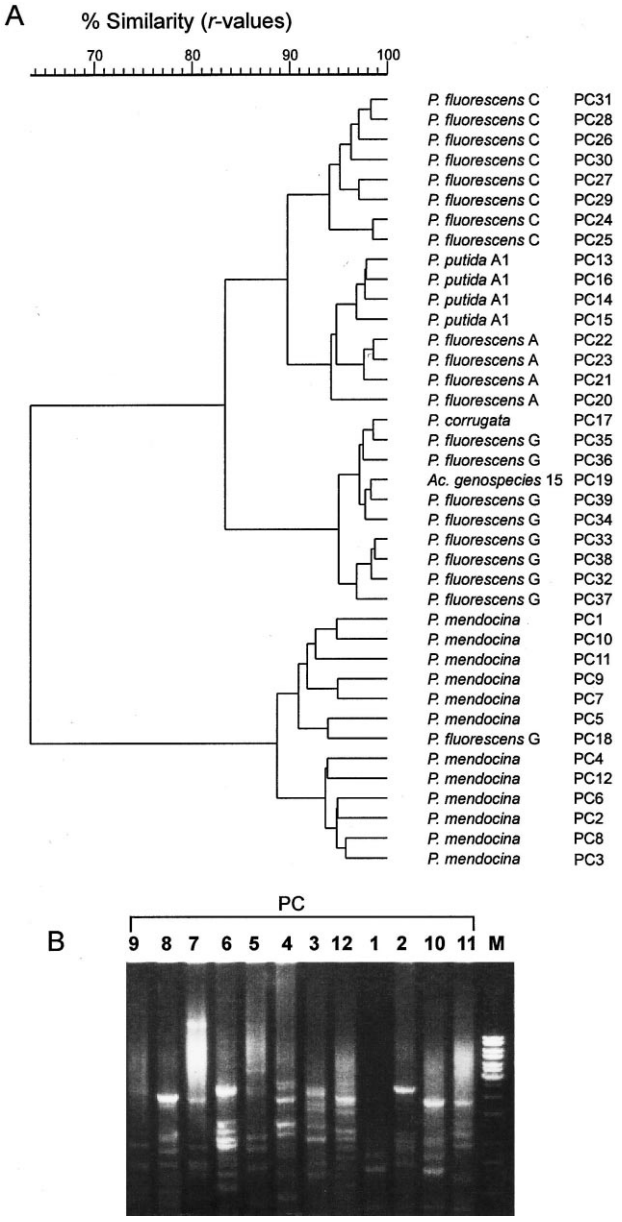

Fig. 2. A: Computer-assisted analysis of rep-PCR genomic fingerprints. Dendrogram shows degrees of similarities of the BOXA1R primer [37] generated rep-PCR genomic fingerprints of 39 isolates. Analysis was performed using the Ward method [32] of neighbor joining of Pearson's product-moment coefficients calculated from the densitometric curves of the fingerprints. On the scale, *r*-values are expressed as percentages. Procedures were performed with GelCompar software (version 4.0; Applied Maths, Kortrijk, Belgium). B: rep-PCR fingerprinting patterns from genomic DNA of *P. mendocina* isolates. Numbers above the lanes refer to the strains of PHE^+p-CRE^+ bacteria. DNA standard ladder (1 kb) is shown in lane M.

(Fig. 2). The only exception was the *P. fluorescens* strain PC18 which clustered into the *P. mendocina* group. The *P. mendocina* cluster was the most heterogeneous. *P. fluorescens* biotypes A, C and G formed separate clusters where biotype A cluster was more related to the *P. putida* cluster. The detection frequency of potential siblings

Table 1
Identification and characterization of phenol- and *p*-cresol-degraders[a]

Strain	Isolation			Plasmid DNA bands	Induction of PCMH with phenol	PCR products with gene primers			Dioxygenases on		Catabolic type
	site	date	media			*phe*A	*xyl*E E1/E2	C23OE2/E3	phenol	*p*-cresol	
P. mendocina strains											
PC1	1	5	BENZ	0	−	−	+	+	C23O$^{xyl/PC1}$	C23O$^{xyl/PC1}$	I
PC2	2	4	PHE	0	−	−	+	+	C23O$^{xyl/PC2}$	C23O$^{xyl/PC2}$	I
PC3	2	3	PHE	2	−	−	+	+	C23O$^{xyl/PC3}$	C23O$^{xyl/PC3}$	I
PC4	5	5	LB	1	−	−	+	+	C23O$^{xyl/PC4}$	C23O$^{xyl/PC4}$	I
PC5	7	5	PHE	0	−	−	+	+	C23O$^{xyl/PC5}$	C23O$^{xyl/PC5}$	I
PC6	6	6	PHE	1	−	−	+	+	C23O$^{xyl/PC6}$	C23O$^{xyl/PC6}$	I
PC7	7	5	SAL	0	−	−	+	+	C23O$^{xyl/PC7}$	C23O$^{xyl/PC7}$	I
PC8	7	5	BENZ	0	−	−	+	+	C23O$^{xyl/PC8}$	C23O$^{xyl/PC8}$	I
PC9	5	6	PHE	0	−	−	+	+	C23O$^{xyl/PC9}$	C23O$^{xyl/PC9}$	I
PC10	1	4	LB	0	−	−	+	+	C23O$^{xyl/PC10}$	C23O$^{xyl/PC10}$	I
PC11	1	3	*m* TOL	0	−	−	+	+	C23O$^{xyl/PC11}$	C23O$^{xyl/PC11}$	I
PC12	6	5	BENZ	0	+	−	−	+	C23O^{PHE}	PC34O^{PHE}	III
P. putida A1 strains											
PC13	1	3	*m* TOL	1	−	+	−	−	C12O^{pheBA}	PC34O^{p-CRE}	II
PC14	1	3	PHE	1	−	−	−	−	C12O^{PHE}	PC34O^{p-CRE}	II
PC15	2	3	*m* TOL	0	−	−	−	−	C12O^{PHE}	PC34O^{p-CRE}	II
PC16	1	3	BENZ	2	−	+	−	−	C12O^{pheBA}	PC34O^{p-CRE}	II
P. corrugata strain											
PC17	1	3	PHE	1	−	+	−	−	C12O^{pheBA}	PC34O^{p-CRE}	II
A. genospecies strain 15											
PC19	5	6	PHE	0	−	−	+	+	C23O$^{xyl/PC19}$	C23O$^{xyl/PC19}$	I
P. fluorescens biotype A strains											
PC20	1	3	*m* TOL	2	−	+	+	+	C23O$^{xyl/PC20}$ C12O^{pheBA}	C23O$^{xyl/PC20}$	I
PC21	1	4	LB	2	+	−	−	+	C23O^{PHE}	PC34O^{PHE}	III
PC22	1	4	LB	2	+	−	−	+	C23O^{PHE}	PC34O^{PHE}	III
PC23	1	3	LB	2	+	−	−	+	C23O^{PHE}	PC34O^{PHE}	III
P. fluorescens biotype C strains											
PC24	8	1	LB	1	−	+	−	−	C12O^{pheBA}	PC34O^{p-CRE}	II
PC25	8	4	*m* TOL	0	−	+	−	−	C12O^{pheBA}	PC34O^{p-CRE}	II
PC26	1	4	LP	1	−	+	−	−	C12O^{pheBA}	PC34O^{p-CRE}	II
PC27	3	6	PHE	0	−	−	+	+	C23O$^{xyl/PC27}$	C23O$^{xyl/PC27}$	I
PC28	6	5	PHE	1	−	−	−	−	C12O^{PHE}	PC34O^{p-CRE}	II
PC29	6	5	SAL	0	−	−	−	−	C12O^{PHE}	PC34O^{p-CRE}	II
PC30	1	3	PHE	1	−	+	−	−	C12O^{pheBA}	PC34O^{p-CRE}	II
PC31	1	4	LB	1	−	+	−	−	C12O^{pheBA}	PC34O^{p-CRE}	II
P. fluorescens biotype G strains											
PC18	1	4	LB	2	+	−	−	+	C23O^{PHE}	PC34O^{PHE}	III
PC32	1	3	LB	2	+	−	−	+	C23O^{PHE}	PC34O^{PHE}	III
PC33	1	3	LB	2	+	−	−	+	C23O^{PHE}	PC34O^{PHE}	III
PC34	1	4	LB	2	+	−	−	+	C23O^{PHE}	PC34O^{PHE}	III
PC35	1	3	PHE	3	+	+	−	+	C23O^{PHE} C12O^{pheBA}	PC34O^{PHE}	III
PC36	3	2	LBCb	1	−	−	+	+	C23O$^{xyl/PC36}$	C23O$^{xyl/PC36}$	I
PC37	1	3	LB	2	+	−	−	+	C23O^{PHE}	PC34O^{PHE}	III
PC38	1	3	BENZ	2	+	−	−	+	C23O^{PHE}	PC34O^{PHE}	III
PC39	2	3	HEPT	0	+	+	−	−	C12O^{pheBA}	PC34O^{PHE}	II

[a]Samples were collected six times (1, 14.09.1993; 2, 30.11.1993; 3, 02.03.1994; 4, 02.06.1994; 5, 15.09.1994; 6, 18.04.1995) from seven sampling sites (see Section 2) and bacteria were isolated on M9 media supplemented with benzoate (BENZ), *m*-toluate (*m* TOL), salicylate (SAL), phenol (PHE), *n*-heptane (HEPT) or on L-broth and L-broth with carbenicillin (1000 μg ml^{-1}). C23O$^{xyl/PC1}$–C23O$^{xyl/PC11}$, C23O$^{xyl/PC19}$, C23O$^{xyl/PC20}$, C23O$^{xyl/PC27}$, C23O$^{xyl/PC36}$, phenol (PHE)- and *p*-cresol (*p*-CRE)-induced C23O of the *xyl*E gene type of the TOL plasmid pWWO; C23O^{PHE}, PHE-induced C23O; C12O^{pheBA}, PHE-induced C12O of the *phe*BA operon; C12O^{PHE}, PHE-induced C12O; PC34O^{CRE}, *p*-CRE-induced protocatechuate 3,4-dioxygenase; PC34O^{PHE}, *p*-CRE-induced PC34O and PHE-induced PCMH; 0,1,2,3, denotes the number of plasmid DNA bands revealed by agarose gel electrophoresis; +, denotes the PCR products with *phe*A, *xyl*E1/E2 and C23OE2/E3 gene primers, and induction of PCMH with phenol; I, denotes the *meta-meta*, II, *ortho-ortho* and III, *meta-ortho* catabolic types.

should reflect the extent of their dominance in water samples. Comparison of our experimental data (Table 1 and Fig. 2) suggests that the isolates belonging to the same cluster were heterogeneous. Even though our results do not exclude the possibility of existence of few siblings among the isolated strains, we were not able to confirm that possibility.

3.3. Prevalence of meta pathway in phenol and ortho pathway in p-cresol degradation

In the majority of PHE$^+$*p*-CRE$^+$ strains (24 isolates), degradation of phenol was performed via the *meta* pathway, as revealed by induction of C23O (Table 1). Degradation of phenol by induction of the *ortho* pathway enzyme C12O was shown only in 13 strains: all four strains of *P. putida*, *P. corrugata* strain PC17, seven out of eight strains of *P. fluorescens* biotype C and biotype G strain PC39. Two strains, PC20 and PC35, expressed both C12O and C23O activities if grown on a phenol medium. In contrast to the predominant nature of phenol catabolism via *meta* fission of catechol, catabolism of *p*-cresol was in most cases (24 isolates) performed via the induction of the protocatechuate *ortho* pathway key enzyme (PC34O). In the remaining 15 strains, degradation of *p*-cresol was performed via the *meta* pathway by the induction of C23O (Table 1). Most of these strains (11) belonged to the species *P. mendocina*, and only four strains were representatives of other species: *A. genospecies* 15 PC19, *P. fluorescens* strains PC20 (biotype A), PC27 (biotype C) and PC36 (biotype G).

3.4. Three main catabolic types in PHE$^+$p-CRE$^+$ strains

On the basis of *meta* and *ortho* fission of the aromatic ring in the degradation of phenol and *p*-cresol, three main catabolic types were found in PHE$^+$*p*-CRE$^+$ strains (Table 1). Type I (*meta-meta* or C23O-C23O), present in 15 strains, was characterized by degradation of both phenol and *p*-cresol via the *meta* pathway through the induction of C23O. This type was distributed most frequently among *P. mendocina* strains (11 strains out of 12). In addition, catabolic type I was also detected in strains PC19, PC27 and PC36. It should be noted that among the strains possessing catabolic type I, *P. fluorescens* biotype A strain PC20 was an exception as it expressed both C23O and C12O activity induced by phenol. Type II (*ortho-ortho* or C12O-PC34O) was characterized as degradation of phenol and *p*-cresol via the *ortho* pathway by the induction of C12O and PC34O, respectively (Table 1). This catabolic type was present in 13 strains belonging to the species *P. putida, P. corrugata* and *P. fluorescens* biotype C and in one strain of biotype G (strain PC39). Catabolic type III (*meta-ortho* or C23O-PC34O) was detected in 11 strains belonging to the species *P. fluorescens* (biotypes A and G) and in one *P. mendocina* strain (PC12). *P. fluorescens* PC35 was different from other members of type III because in this strain the activity of C12O was also induced by phenol.

3.5. Distribution of xylE genes among the catabolic type I PHE$^+$p-CRE$^+$ strains

The colony hybridization experiments with specific *xyl*E gene primers (E1 and E2) revealed 15 positive strains (data not shown). The same 15 strains gave the PCR product with C23O gene primers E1/E2 and E2/E3 (Table 1 and Fig. 3B). These strains belonged, without exception, to catabolic type I, in which the degradation of both phenol and *p*-cresol was performed via the *meta* pathway by the induction of C23O (Table 1). From these 15 strains 11 belonged to the species *P. mendocina* (PC1–PC11), three

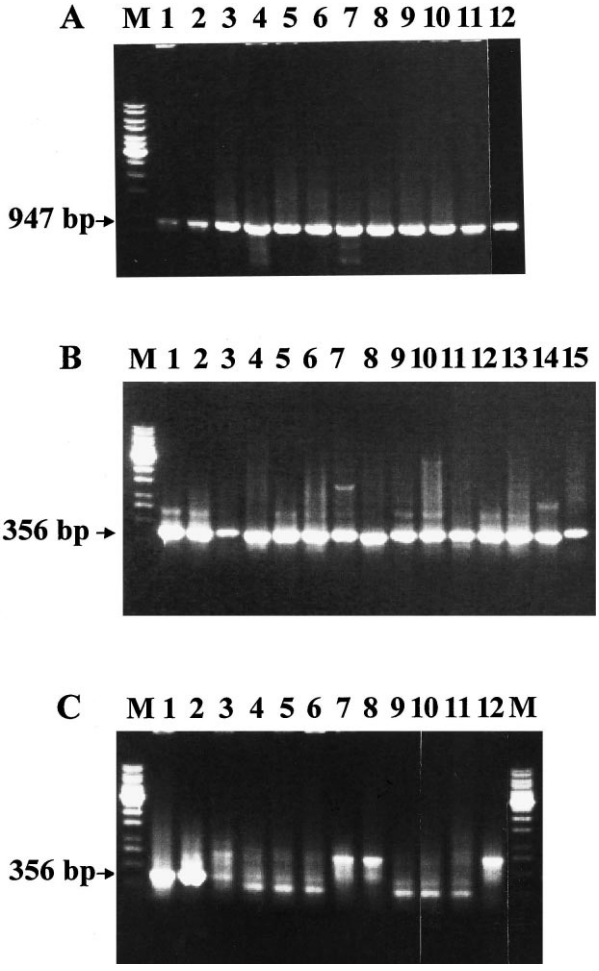

Fig. 3. PCR detection of the *phe*A (A) and C23O genes (B and C) in PHE$^+$*p*-CRE$^+$ strains. Numbers above the lanes refer to the strain of PHE$^+$*p*-CRE$^+$ bacteria. A: Lanes: 1, PC35; 2, PC24; 3, PC20; 4, PC16; 5, PC25; 6, PC13; 7, PC39; 8, PC26; 9, PC30; 10, PC17; 11, PC31; 12, EST1026 as a positive control. B: Lanes: 1, E2 as a positive control; 2, PC1; 3, PC20; 4, PC2; 5, PC3; 6, PC4; 7, PC5; 8, PC6; 9, PC7; 10, PC8; 11, PC9; 12, PC11; 13, PC10; 14, PC36; 15, PC27. C: Lanes: 1, E2 as a positive control; 2, PC12; 3, PC35; 4, PC37; 5, PC23; 6, PC21; 7, PC32; 8, PC33; 9, PC38; 10, PC34; 11, PC22; 12, PC18. DNA standard ladder (1 kb) is shown in lane M.

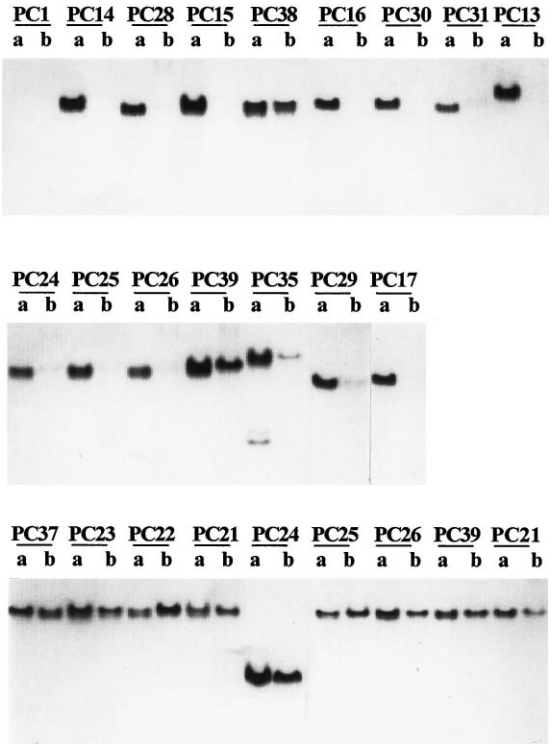

Fig. 4. Polyacrylamide gel electrophoresis of PCMHs. The samples for nondenaturing PAGE were crude extracts from *p*-cresol- (a) and phenol-grown (b) cells of PHE$^+$*p*-CRE$^+$ strains. Onto each lane 10 μg of protein was loaded. The strain PC1 was used as a negative control.

to the species *P. fluorescens* (PC20, PC27 and PC36) and one was *A. genospecies* 15 PC19. The other 11 strains with C23O genes belonged to catabolic type III and yielded PCR products of different size only with C23O-gene primers E2 and E3 (Table 1 and Fig. 3C). The majority of these strains (PC21, PC22, PC23; strains PC34, PC37, PC38) yielded a PCR product smaller than the PCR product of the *xyl*E gene of the control strain E2. The other strains (PC18, PC32, PC33, PC35 and PC12), in contrast, yielded a PCR product with a higher molecular mass. Enzymological assays revealed that the C23O activity was present in all observed 26 strains carrying C23O genes.

3.6. Distribution of the pheA gene among the catabolic type II PHE$^+$p-CRE$^+$ strains

Of 39 PHE$^+$*p*-CRE$^+$ bacteria, 11 strains identified as *P. putida* A1 (PC13 and PC16), *P. corrugata* (PC17), *P. fluorescens* biotype C (PC24-PC26, PC30 and PC31), biotype A (PC20) and biotype *G* (PC35 and PC39), yielded a specific *phe*A-PCR product (Table 1 and Fig. 3A). All except PC20 were type II but four strains from catabolic type II did not generate a specific *phe*A-PCR product (Table 1).

3.7. Induction of p-cresol pathway enzyme PCMH by phenol in the catabolic type III PHE$^+$p-CRE$^+$ strains

The 24 strains degrading *p*-cresol via the protocatechuate *ortho* pathway, by induction of PC34O, showed induction of PCMH activity, the first enzyme of *p*-cresol catabolism via the protocatechuate pathway (Fig. 4). Nondenaturing PAGE of crude extracts from *p*-cresol-grown cells of 20 strains revealed similar migration of reactive proteins and four (PC12, PC35, PC17 and PC29) had PCMH with a different relative migration (Fig. 4). It is interesting to note that among the above-mentioned 24 strains, 11 type III strains, degrading phenol via the *meta*-pathway by the induction of C23O, were able to induce PCMH also during the growth on phenol (Fig. 4). Therefore, it was revealed that in all type III strains, phenol was the inducer not only for *meta* pathway enzymes but also for *p*-cresol *ortho* pathway enzymes. Among the other 13 strains there was only one exceptional strain, PC39, in which both PCMH and C12O were induced by phenol (Table 1 and Fig. 4).

3.8. Occurrence of plasmid DNA

The presence of plasmid DNA was revealed for 24 strains (61.5%) (Table 1). Plasmid profiles with two or more plasmid replicons were visualized in the catabolic type III strains except in PC12, that did not carry a plasmid. The type II strains that contained the *phe*A gene had one plasmid replicon with the exception of strain PC25 and PC39. The other type II strains, which had no *phe*A gene, did not contain any plasmid. We could not detect any plasmid DNA in the strains of catabolic type I, except in PC3, PC4, PC6, PC20 and PC36 (Table 1). In 11 strains out of 26 having the C23O activity no plasmids were detected. Thus, the presence or absence of plasmid DNA reflects heterogeneity of PHE$^+$*p*CRE$^+$ isolates (Table 1).

3.9. Induction of phenol- and p-cresol-degrading enzymes in representative strains of the three catabolic types

For further characterization of the three catabolic types, we chose one representative strain from each type: *P. mendocina* PC1 (type I or C23O-C23O type), *P. fluorescens* C PC24 (type II or C12O-PC34O type) and *P. fluorescens* G PC18 (type III or C23O-PC34O type). The representative strains did not reveal differences in the induction of PC34O and POBH by POB (Table 2). Hence, these strains harbor a functional protocatechuate pathway. Benzoate, as a typical substrate for the *ortho* pathway, was metabolized in all three catabolic types via the catechol *ortho* pathway by the induction of C12O. However, the strains PC18 and PC24 had two additional characteristic features. First, benzoate caused an unexpectedly low level of induction of C12O after 4 h of growth on the CAA medium (Table 2). Secondly, POBH and PC34O in strain PC18, and PC34O in strain PC24 were induced moderately by benzoate and phenol. Thus, it is possible that benzoate and phenol could favor the induction of the protocatechuate branch of the *ortho* pathway. In the strain PC1, C12O

Table 2
Expression of key catabolic enzymes in crude extracts of phenol- and *p*-cresol-degrading bacteria grown on different substrates

Strain	Substrate(s) of growth[b]	Sp. act. (nmol min^{-1} mg^{-1} of protein) of enzymes[a]				
		PMO	C12O	C23O	PC34O	POBH
PC1	CAA	< 1	< 1	< 1	< 1	< 1
	PHE	< 1	33 ± 7	694 ± 72	< 1	< 1
	CAA+PHE	< 1	14 ± 6	1 020 ± 98	9 ± 3	2 ± 1
	p-CRE	< 1	< 1	535 ± 42	< 1	< 1
	CAA+*p*-CRE	< 1	3 ± 2	197 ± 13	4 ± 2	2 ± 1
	BENZ	< 1	304 ± 22	14 ± 6	< 1	< 1
	CAA+BENZ	< 1	262 ± 23	75 ± 10	54 ± 5	12 ± 6
	CAA+POB	< 1	40 ± 8	2 ± 2	843 ± 65	186 ± 17
PC18	CAA	< 1	< 1	< 1	< 1	< 1
	PHE	< 1	< 1	758 ± 68	71 ± 19	26 ± 8
	CAA+PHE	< 1	12 ± 4	97 ± 11	3 ± 1	1 ± 1
	p-CRE	< 1	< 1	< 1	390 ± 28	260 ± 22
	CAA+*p*-CRE	< 1	14 ± 3	< 1	287 ± 29	108 ± 18
	BENZ	< 1	318 ± 25	< 1	237 ± 21	190 ± 16
	CAA+BENZ	< 1	12 ± 5	< 1	9 ± 2	25 ± 4
	CAA+POB	< 1	12 ± 10	< 1	919 ± 83	267 ± 27
PC24	CAA	< 1	< 1	< 1	< 1	< 1
	PHE	79 ± 11	1 230 ± 118	< 1	143 ± 18	7 ± 2
	CAA+PHE	153 ± 15	419 ± 17	< 1	70 ± 6	8 ± 3
	p-CRE	< 1	< 1	< 1	770 ± 54	89
	CAA+*p*-CRE	8 ± 3	20 ± 6	< 1	121 ± 20	51 ± 14
	BENZ	26 ± 12	1 230 ± 124	< 1	89 ± 14	32 ± 7
	CAA+BENZ	53 ± 6	82 ± 12	< 1	83 ± 14	5 ± 4
	CAA+POB	2 ± 1	18 ± 8	< 1	159 ± 18	115 ± 15
E2	CAA	< 1	< 1	< 1	< 1	< 1
	PHE	< 1	< 1	1 340 ± 117	< 1	< 1
	p-CRE	< 1	< 1	1 680 ± 97	< 1	< 1
	BENZ	< 1	< 1	2 100 ± 88	< 1	< 1
EST1026	PHE	464 ± 34	6 430 ± 212	< 1	< 1	< 1
	BENZ	78 ± 10	3 730 ± 234	< 1	< 1	< 1

[a]Specific activity values of enzymes are means of four independent experiments ± standard errors of the means.
[b]Mid-log growth phase cells of strains grown on a single substrate were used. In the case of induction experiments, cells were collected 4 h after the induction.

was induced by benzoate, but the cells grown on either phenol, *p*-cresol or benzoate revealed no expression of PCMH, POBH or PC34O, suggesting that both phenol and *p*-cresol were degraded via the *meta* pathway through the induction of C23O (Table 2). In the laboratory construct *P. putida* AC783 E2 [28], both catechol and *p*-cresol *ortho* pathways are nonfunctional and the genetic design of this strain provides an opportunity to degrade efficiently not only phenol and *p*-cresol but also benzoate via the *meta* pathway by the induction of C23O (Table 2). In strain PC24, C12O and PMO were both induced by phenol and benzoate, suggesting that these genes were linked (Table 2). The same type of induction was also found in the standard strain *P. putida* PaW85 pEST1026, which degrades phenol due to the *phe*BA operon.

4. Discussion

In a previous study phenol-degraders were common suggesting their selection under permanent phenol and *p*-cresol contamination [25]. According to the HPLC analysis the leachate contained mainly phenol (up to 70 mg l^{-1}) and *p*-cresol (up to 40 mg l^{-1}). The microbial community should adapt to the pollutants present in a continuously polluted environment, and thus it was expected that the PHE^+ bacteria would also be able to mineralize *p*-cresol. Because of the long-term selective pressure metabolic types will be selected with better chances of survival. It could be expected also that a few microbial species with the most favorite catabolic type would be predominant. In fact, the present study did not support these assumptions. We found three main catabolic types of degradation of phenol and *p*-cresol among 39 PHE^+*p*-CRE^+ newly isolated strains: 15 strains of *meta-meta* (C23O-C23O); 13 strains of *ortho-ortho* (C12O-PC34O) and 11 strains of *meta-ortho* (C23O-PC34O) type. We did not find strains of *ortho-meta* catabolic type, in which phenol and *p*-cresol are degraded by C12O and C23O, respectively. This is in contradiction with the general assumption that phenol, as a nonsubstituted aromatic compound, should be degraded via the *ortho* pathway in phenol-degrading bacteria, whereas *p*-cresol (4-methylphenol), an alkyl-substituted ar-

omatic compound, should be catabolized via the *meta* pathway in *p*-cresol-degraders [42]. However, in PHE$^+$*p*-CRE$^+$ bacteria the absence of the *ortho-meta* (C12O-C23O) type revealed selective limitation for one of the two possible metabolic alternatives. This can easily be explained on the basis that catabolism of phenol and *p*-cresol must use two different pathways for aromatic ring fission of catechol. It is well known that the *ortho* and *meta* fission of catechol are metabolic alternatives and the use of one or the other depends on the nature of the growth substrates as well as on the microbial species [43].

In the present study we showed that PHE$^+$*p*-CRE$^+$ bacteria must have certain specific features at the level of genetic rearrangement, regulation of biochemical pathways, and even at the species level. The number of strains in each of the three catabolic types was almost the same and therefore we were interested in the species pattern of these types. The results of the taxonomic study of PHE$^+$*p*CRE$^+$ bacteria showed the predominance of *P. mendocina* in the *meta-meta* type, *P. putida* and biotype C of *P. fluorescens* in the *ortho-ortho* type and biotypes A and G of *P. fluorescens* in the *meta-ortho* type (Table 1). Considering that rep-PCR fingerprints may reflect strain-specific genomic structure [44], these results show that, with few exceptions, a majority of isolates represents a unique strain. The rep-PCR genomic fingerprinting with the BOXAIR primers clearly clustered not only different *Pseudomonas* species but also different *P. fluorescens* biotypes into distinct groups and verified that *P. fluorescens* biotypes were even more different than different *Pseudomonas* species (Fig. 2). The results obtained from the cluster analysis of the rep-PCR fingerprinting patterns performed by GelCompar program show that the BIOLOG GN species identification, in fact, characterizes grouping of new isolates belonging to the same species or *P. fluorescens* biotypes (Fig. 2).

In the catabolic type I bacteria only of *meta* cleavage type of aromatic ring were found. The degradation of phenol via the *meta* pathway has been described by many authors. For example, plasmid-encoded multicomponent phenol hydroxylases, determined by *dmp*LMNOP [15] and *phl*BCDEF [17] operons, which, being evolutionary conserved, have *meta* pathway genes in different transcriptionally fused combinations. A non-transcriptionally fused combination of *meta* pathway genes seems to be present in the *tbu*D-encoded phenol/*p*-cresol hydroxylase [19]. The most frequent genetic pathway for phenol degradation via the *meta* pathway related to the *dmp*KLMNOP genes was observed among phenol-degrading marine isolates [15] and in the isolates from river water contaminated with the oil shale industry leachate [24]. In the case of incompatible substrates, their efficient mineralization could be achieved by the induction of a single type of aromatic ring cleavage. This situation is common in the case of degradative plasmids determining the utilization of phenolic compounds via catechol, where only a single type of aromatic ring cleavage (*meta* or *ortho*) can be used depending on the structure of the aromatic compound [3].

We found that catabolic type I was distributed mainly among the strains of *P. mendocina* and that they carried the *xyl*E type genes for C23O (Table 1 and Fig. 3). Cluster analysis of genomic fingerprints revealed that strains of this species formed a heterogeneous group (Fig. 2). Thus, the wide distribution of catabolic type I among *P. mendocina* strains should reflect the existence of the genetic background necessary for the proliferation of type I strains under strong selective pressure. In certain cases other pseudomonads can also have this type of catabolism. For example, a laboratory constructed strain *P. putida* E2 does not contain a functional *ortho* pathway but, due to particular genetic rearrangements, it uses not only phenol and *p*-cresol, but also benzoate via the *meta* pathway by very strong induction of C23O (Table 2).

p-Cresol catabolism via the *ortho* pathway in catabolic type II (C12O-PC34O) strains bypasses catechol by using the protocatechuate branch. The presence of PCMH in *p*-cresol-grown cells also indicates that degradation of this substrate can occur through the protocatechuate branch (Fig. 4). Thirteen PHE$^+$*p*-CRE$^+$ bacterial strains from catabolic type II belonged to three different species and to three different biotypes of *P. fluorescens* (Table 1). Therefore, this group of strains has probably not evolved on the basis of the genetic potential of a particular species. We have shown recently that the deliberately released *phe*BA operon determining the synthesis of PMO and C12O was transferred in the same study area to indigenous bacteria most probably due to horizontal gene transfer, enriching the natural genetic variety of bacteria for biodegradation [24]. The *phe*A gene was detected in 9 PHE$^+$*p*-CRE$^+$ bacterial strains belonging to type II (Table 1 and Fig. 3A). Therefore, we suppose that in the catabolic type II strains, phenol catabolism through the catechol *ortho* pathway is mostly determined by a *phe*BA operon.

The *meta* pathway of the type III strains is different to that of type I strains. We found that C23O genes of type III strains yielded PCR products only with primers E2 and E3 (Fig. 3C). The characteristic feature for type III strains was that during the growth on phenol media all strains had PCMH activity. We suppose that induction of the *p*-cresol protocatechuate pathway by phenol reflects adaptation of bacteria to degradation of *p*-cresol in the environment. It is also possible that a plasmid DNA can promote establishment of catabolic type III strains, because nearly all of them had plasmid DNA (Table 1).

Conservation of central pathways (i.e. *meta*- or *ortho*-pathways) has led to the idea that catabolic pathways are built up by the acquisition of pre-evolved 'metabolic modules' [45]. Nordlund et al. [15] demonstrated conservation of structural and regulatory genes involved in phenol catabolism within the strains isolated from diverse geographical locations (UK, Norway and USA) and from a wide range of habitats including activated sludge, sea water and

fresh-water mud. The most frequent genetic pathway for phenol degradation was related to the multicomponent phenol hydroxylase genes linked to the C23O gene. On the other hand, Peters et al. [24] indicated that released genetic material (*phe*BA operon encoding PMO and C12O) could enrich the natural genetic variety for biodegradation, if it encodes a beneficial capability. Up to now the *p*-cresol pathways have been characterized only in few environmental isolates [10–12,40] and, as a rule, they are not connected with phenol degradation. It is also important to stress that it is not yet clear whether laboratory data can be extrapolated to predict and explain the growth properties and activity of bacteria in the natural environment [46].

The present study showed that specific catabolic types of PHE^+p-CRE^+ bacteria, isolated from the natural bacterial assemblage being continuously under strong selective pressure favor utilization of nonsubstituted and methyl-substituted phenols. Further research concerning mixed substrate cultivation of the representatives of the three catabolic types of the PHE^+p-CRE^+ bacteria is planned to elucidate their biodegradation efficiency.

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