ELSEVIER

FEMS Microbiology Ecology 37 (2001) 79–89

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Reversible accumulation of *p*-hydroxybenzoate and catechol determines the sequential decomposition of phenolic compounds in mixed substrate cultivations in pseudomonads

Eeva Heinaru [a,*], Signe Viggor [b], Eve Vedler [a], Jaak Truu [a], Merike Merimaa [a], Ain Heinaru [a]

[a] *Department of Genetics, Institute of Molecular and Cell Biology, University of Tartu, Riia 23, 51010 Tartu, Estonia*
[b] *Department of Environmental Technologies, Centre of Strategic Competence, University of Tartu, Riia 23, 51010 Tartu, Estonia*

Received 5 March 2001; received in revised form 12 June 2001; accepted 13 June 2001

First published online 12 July 2001

Abstract

Accumulation of key catabolic intermediates during degradation of phenol, *p*-cresol and benzoate was studied in two-substrate batch cultivations by the strains *Pseudomonas mendocina* PC1, *Pseudomonas fluorescens* PC18 and *P. fluorescens* PC24. According to sequence analysis of 16S rRNA genes the strains belonged to different monophyletic clusters of *Pseudomonas*. The catechol 2,3-dioxygenase (C23O) gene, *xylE*, of strain PC1 and the phenol monooxygenase gene, *pheA*, of PC24 were localised on the chromosome, while the C23O gene, *xylE*, of strain PC18 and the *p*-cresol methylhydroxylase gene, *pchF*, of strains PC18 and PC24 were on plasmids. It was shown that, if the substrates were degraded from mixtures using either catechol *meta*, catechol *ortho* or catechol *ortho* and protocatechuate *ortho* pathways, then both substrates were catabolised simultaneously (nondiauxic growth) without the accumulation of intermediates. Exceptionally, degradation of phenol and benzoate via the catechol *ortho* pathway caused irreversible accumulation of *cis*,*cis*-muconate without detectable effect on simultaneous consumption of substrates. When the substrates were degraded from mixtures through *meta* and *ortho* catabolic pathways, the sequential consumption of substrates (diauxic growth) was observed due to the reversible accumulation of the catabolic intermediates *p*-hydroxybenzoate or catechol. Regulation of parallel catabolic pathways by the accumulation of catabolic intermediates depended on the concentration of growth substrates. At low concentrations simultaneous degradation occurred and the antagonistic effect of *p*-hydroxybenzoate on the degradation of phenol was diminished. In strain PC18 only the accumulation of *p*-hydroxybenzoate during growth on a phenol–*p*-cresol mixture seems to be directly metabolically regulated because phenol also induces the catabolic pathway for *p*-cresol degradation. Partial sequencing of the *pchF* genes of strains PC18 and PC24 showed considerable differences.

Keywords: Pseudomonad; Phenol-degrading bacterium; *p*-Cresol-degrading bacterium; 16S rRNA; *p*-Cresol methylhydroxylase; Accumulation of *p*-hydroxybenzoate; Catechol and *cis*,*cis*-muconate

1. Introduction

The study of microbial activities under mixed-substrate conditions is the starting point for several biotechnological applications [1]. Although certain mixtures are degraded more rapidly than compounds present individually [2], the biodegradation rate of aromatic mixtures may be much lower under natural conditions, mostly due to catabolite repression, but also for other reasons [3–5].

In *Pseudomonas*, genes encoding the enzymes involved in catabolism of aromatic compounds are not expressed if certain other growth-supporting substrates such as succinate, lactate, citrate, pyruvate, acetate, glutamate, gluconate, glucose [6,3,4,7] or benzoate [8] are available. Plasmid-encoded degradative pathways for aromatics may also be subject to catabolite repression by organic acids. It was found that organic acids (e.g., succinate, lactate, acetate) and carbohydrates (e.g., glucose and gluconate) can repress phenol degradation in *Pseudomonas putida* H [5]. In this case catabolite repression control was achieved at the transcriptional level of plasmid-encoded *phl* genes. It was also shown that the *meta* cleavage pathway was less susceptible to catabolite repression [3]. However, little is

* Corresponding author. Tel.: +37 (27) 37 50 14; Fax: +37 (27) 42 02 86.
E-mail address: eheinaru@ebc.ee (E. Heinaru).

0168-6496/01/$20.00
PII: S0168-6496(01)00147-7

known about molecular mechanisms of catabolite repression in pseudomonads, which may be different to that in *Escherichia coli*. Only recently the involvement of regulatory protein Crc in the control of catabolite repression of the *bkd* operon of *Pseudomonas aeruginosa* and *P. putida* has been described [9].

The β-ketoadipate or *ortho* pathway consists of two parallel branches for the catabolism of catechol and protocatechuate, derived from benzoate and *p*-hydroxybenzoate (POB), with catechol 1,2-dioxygenase (C12O) and protocatechuate 3,4-dioxygenase (PC34O) being the respective key enzymes [10]. The *meta* pathway for the catabolism of catechol is catalysed by catechol 2,3-dioxygenase (C23O), and the ring cleavage of methyl-substituted aromatic compounds occurs generally through this pathway [11]. An alternative catabolic route has been described for methylated phenols in which catabolism is initiated by the oxidation of a methyl group, followed by conversion of the ring cleavage product of protocatechuate to β-ketoadipate. The first enzyme for the degradation of *p*-cresol and dimethylphenols via the protocatechuate branch of the β-ketoadipate pathway, *p*-cresol methylhydroxylase (PCMH), is a flavocytochrome *c* consisting of two subunits of equal size [12]. PCMH has a limited substrate range and requires an alkyl-substituted phenolic ring with a hydroxyl group in the *para* position [13]. In *Pseudomonas*, degradation of *p*-cresol also occurs via the catechol *meta* pathway, phenol can be degraded by both catechol *ortho* and *meta* pathways, and benzoate preferably by the catechol *ortho* pathway [14].

The *ortho* and *meta* pathways are alternatives, because simultaneous operation of these pathways yields toxic intermediates and causes accumulation of dead-end metabolites [15,11]. During batch growth of *Ralstonia eutropha* on a benzoate–phenol mixture, benzoate completely inhibited phenol degradation causing diauxic growth, and repression of phenol utilisation was attributed to the presence of benzoate [16]. Benzoate was also the preferred substrate during growth of the same bacteria on a benzoate–acetate mixture [17]. In this case, the actual trigger of the repression signal was not benzoate, but catechol, transiently accumulating in the medium when high specific rates of benzoate consumption were reached [17,14]. It was also shown that *P. putida* cells degrade benzoate in preference to POB by repressing POB transport by transcriptional downregulation of *pcaK*, the gene encoding POB permease [8,18]. In *Acinetobacter calcoaceticus*, benzoate degraded via the catechol branch was consumed in preference to POB degraded via the protocatechuate branch when both compounds were simultaneously present [19]. However, in this case the product of catechol ring cleavage, *cis*,*cis*-muconate, inhibited utilisation of POB in the presence of benzoate. On the other hand, if an organism can convert a compound present in the environment into a metabolite of an existing catabolic pathway, it will gain a selective advantage by being able to use this compound as a nutrient [20].

Microorganisms tend to be more competitive in using complex mixtures since they have already survived natural selection [21]. The results of our previous work demonstrate a 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 river water continuously polluted with phenolic compounds of oil shale leachate [22]. In this work three main catabolic types (*meta-meta*, *ortho-ortho* and *meta-ortho*) for the degradation of phenol and *p*-cresol were revealed. The *meta-meta* catabolic type strains use *meta* cleavage of catechol by C23O when grown on phenol and *p*-cresol. The *ortho-ortho* catabolic type strains degrade phenol through *ortho* fission of catechol by C12O, and *p*-cresol through *ortho* cleavage of protocatechuic acid by PC34O. The *meta-ortho* catabolic type strains degrade phenol by using C23O, and *p*-cresol via the protocatechuate *ortho* pathway by the induction of PC34O [22]. The goal of the current investigation was to determine the mechanisms regulating degradation of phenol, *p*-cresol and benzoate from their mixtures, using one representative *Pseudomonas* strain from each catabolic type as a model, and to clarify the role of metabolic intermediates in this process.

2. Materials and methods

2.1. Bacterial strains

The strains *Pseudomonas mendocina* PC1, *Pseudomonas fluorescens* PC24 and *P. fluorescens* PC18 are representatives of different catabolic types and they degrade phenol and *p*-cresol via *meta-meta*, *ortho-ortho* and *meta-ortho* pathways, respectively [22]. Strain PC1 uses *meta* cleavage of catechol by C23O encoded by the TOL plasmid pWWO *xylE* type gene when grown on phenol and *p*-cresol. Strain PC24 contains the *pheBA* operon determining C12O and phenol monooxygenase (PMO). In strain PC18, phenol also induces the protocatechuate *ortho* pathway as revealed by the induction of PCMH. The laboratory-constructed, phenol-degrading strain *P. putida* E2 contains a large, 83-kb plasmid pWWO^{mut90} that carries the TOL plasmid pWWO *meta* cleavage operon [23]. In this strain, both catechol and protocatechuate *ortho* pathways are non-functional. The reference strain for the measurement of the activity of PMO was *P. putida* EST1026 (pEST1026) [24].

2.2. Medium and culture conditions

Filter-sterilised single substrates and their mixtures were supplied as defined sources of carbon and energy in liquid

mineral medium [22]. Final concentrations of substrates in the medium were: 1.3 mM *p*-cresol, 5 mM benzoate, 2.5 mM phenol and 2.5 mM POB. Liquid cultures were grown in 150 ml Erlenmeyer flasks containing 50 ml of mineral medium supplemented with appropriate substrates at 30°C on a rotary shaker. Precultures were inoculated from frozen stocks and, in all cases, the cells were pregrown aerobically on phenol medium to late exponential phase.

Batch culture studies at low concentrations of phenolic compounds (final concentration of each compound 0.6 mg l^{-1}) were performed in 250 ml hermetically closed dark bottles at 20°C. The media in the bottles (approximate oxygen concentration in media 9 mg l^{-1}) were inoculated with cells pregrown on phenol at an initial cell concentration of $2–3\times10^3$ CFU ml^{-1}.

2.3. Analytical measurements

Growth was monitored by optical density measurements at 580 nm ($OD_{580\,nm}$) of at least two independent cultures or by determining cell number by plating. For the enumeration of bacteria, serial dilutions of cultures in 0.9% (w/v) NaCl were plated out onto phenol (2.5 mM) minimal medium in triplicate. Counts were obtained after 5 days of incubation at 30°C. After inoculation, samples were periodically withdrawn from the duplicate shake flask cultures during cell growth. All samples for substrate analysis were filtered through a 0.22-μm pore size Millipore filter and stored frozen (−20°C) until analysis. Substrates and metabolites were analysed using high-performance liquid chromatography (HPLC). HPLC analyses of phenolic compounds were performed using a Kontron Instruments HPLC system 500 (BIO-TEK® Instruments, Inc.) with an automatic injector (Autosampler 560), a binary pump with high pressure gradient mixer (Pump System 525) and an UV diode array detector (Diode Array Detector 540). Separation of phenolic compounds was achieved using a Hypersil ODS (250×4.6 mm i.d., 5 μm) analytical column and a Superspher® 100 RP-18 (10×2 mm i.d., 4 μm) pre-column from Hewlett-Packard (Germany). Elution was carried out at a flow rate of 1 ml min^{-1} with the mobile phase 0.005 M aqueous phosphoric acid–acetonitrile (64:36). Separation was carried out at room temperature. The volume of sample injected by automatic injection was 20 μl. Compounds were detected by measuring UV absorption at 275 nm. Identification of phenolic compounds was performed comparing the UV spectra and retention times with those of reference substrates. Substrates were quantified by calibration with standards. For the registration and calculations of HPLC data the programme KromaSystem2000 was used.

Measurement of oxygen uptake was performed with an oxygen meter (Oxygen sensor CellOx 325, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany).

2.4. Enzyme activity assay

Cultures for enzyme assay were harvested in late exponential growth phase. Crude extracts were prepared and enzyme activity assays were performed as described previously [22]. PMO and *p*-hydroxybenzoate hydroxylase (POBH) activities were determined spectrophotometrically in 50 mM phosphate buffer (pH 7.5) by monitoring disappearance of NADPH at 340 nm as described in [25] and [7], respectively. Activities of C12O and C23O were measured spectrophotometrically by monitoring formation of reaction products at 260 and 375 nm, respectively [26,27]. PC34O activity was measured by determining the decrease of absorption at 290 nm due to the oxidation of protocatechuate [28]. Protein was measured by the method of Bradford [29] with bovine serum albumin as a standard.

2.5. DNA manipulations and sequence analysis

Genomic DNA preparations and other DNA manipulations were carried out by established procedures [30]. Plasmid DNA was detected by the procedures of Connors and Barnsley [31]. 16S rRNA genes of bacterial strains were PCR-amplified using the forward primer PCRI (5′-AGAGTTTGATCATGGCTCAG-3′), complementary to *E. coli* 16S rRNA gene positions 6–26, and the reverse primer PCRII (5′-TACGGTTACCTTGTTACGACTT-3′), complementary to *E. coli* 16S rRNA gene positions 1513–1492. The PCR product (about 1.5 kb) was sequenced with the T7 Sequenase v2.0 PCR product sequencing kit (Amersham Life Science, Inc., 1997), using primers PCRI, PCRII and the internal reverse primers SEQ1 (5′-GTATTACCGCGGCTGCTGG-3′), SEQ2 (5′-TTGCGCTCGTTCGGGACT-3′), SEQ3 (5′-ACGGGCGGTGTGTACAAG-3′) and S4 (5′-CCAGGGTATCTAATCC-3′), complementary to conserved regions of different eubacterial 16S rRNA genes [32].

DNA probes specific to the PCMH gene were amplified as described by Kim et al. [33] by PCR using the following primer pairs: PCHF1, 5′-CTGCAGTTGGTGCCTTACAACAAGATCATGATG-3′ and PCHF2, 5′-CACGAATTCCATGCCGCACTGCATCATGAA-3′ or PCHC1, 5′-GGAATTCTATGACAAGGTTTGFGGCCATTGCCAC-3′ and PCHC2, 5′-GGAATTCATAGGACGCCGGGAAFGCCGGCAT-3′. Amplification was performed in a PCR thermocycler (Biometra, Eppendorf) using 200 ng of chromosomal DNA as a template. The PCR mixture contained 1 U of *Taq* polymerase (Amersham Pharmacia Biotech, Inc.), 5 μl of *Taq* polymerase buffer (10×), 1 μl of nucleotide mixture (dATP, dCTP, dGTP and dTTP; 10 mM of each), 5 μl $MgCl_2$ (25 mM), 2 μl of primers (30 pmol μl^{-1}), template DNA and H_2O added to yield a total reaction volume of 50 μl. The following thermocycling programme was used: initial denaturation at 96°C for 1 min, followed by 30 cycles of 94°C for 45 s, 54°C for

45 s, and 72°C for 60 s. This was followed by a final extension step at 72°C for 8 min.

The amplified DNA products were purified from 2% agarose gels using the QIAquick gel extraction kit (Qiagen Inc.). The nucleotide sequences of *pchF* genes were determined directly from the PCR fragments. DNA sequencing was carried out on an ABI PRISM™ 377 DNA sequencer (Perkin-Elmer) using the DYEnamic™ ET terminator cycle sequencing premix kit (Amersham Pharmacia Biotech, Inc.).

Southern hybridisation analyses were performed as described in Sambrook et al. [30]. Total genomic DNA was digested with *Sma*I or *Eco*RI and separated electrophoretically on a 1% (w/v) agarose gel. Plasmid DNA and restriction fragments of total DNA were transferred to a Hybond-N nylon membrane (Amersham Pharmacia Biotech, Inc.) using a vacuum blotter (Appligene, France). The blot was hybridised with radioactively labelled gene probes, obtained by PCR as 356-bp *xylE* gene products from strains PC1 and PC18 [22], 947-bp *pheA* gene product from strain PC24 [22] and 450-bp *pchF* gene products from strains PC18 and PC24. The amplified products were purified from agarose gels and subsequently random-prime labelled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech, Inc.) using a DNA labelling kit (DecaLabel™, MBI Fermentas). Autoradiograms were generated using PhosphorImager (Molecular Dynamics).

2.6. Phylogenetic analysis and nucleotide sequence accession numbers

The partial sequences of 16S rRNA genes were compared with sequences obtained from the Ribosomal Database Project (RDP), version 7.0 [34], using the SIMILARITY_RANK programme to obtain S_{ab} values with database, and from 16S rDNA sequence data deposited in GenBank using BlastN search. Alignment of 16S rRNA gene sequences was performed using CLUSTAL W [35]. Phylogenetic analyses were performed using the Kimura two-parameter model for nucleotide change and a transition/transversion ratio of 2.0. The phylogenetic tree was reconstructed from the evolutionary distance matrix by the neighbour-joining method [36] using the software package PHYLIP version 3.5 [37]. Bootstrap analyses, which included 100 replicate resampled data sets, were used to estimate the relative confidence in monophyletic groups. The approx. 1500-bp sequences of the 16S ribosomal genes of strains PC1, PC18 and PC24 have been deposited in the GenBank data library with accession No. AF232713, AF228366 and AF228367, respectively.

3. Results

3.1. Phylogeny of the strains investigated

The morphological, physiological and genetic characteristics of strains PC1, PC18 and PC24 identified them as members of the genus *Pseudomonas* [22]. The 16S rRNA gene sequences of pseudomonads (approx. 1500 bp) were adopted in phylogenetic analysis and the resulting phylogenetic tree is shown in Fig. 1. The studied strains belong to different monophyletic clusters of the *Pseudomonas* group. *P. fluorescens* PC18 biotype G was located in the *P. putida* cluster, strain PC24 belonged to a cluster which is different from the *Pseudomonas pseudoalcaligenes–P. mendocina group*. The third group includes *P. mendocina* PC1 and the level of homology between this strain and *P. fluorescens* species was 96%. The level of homology between strains PC18 and PC24 was 99%. The strain most similar to *P. fluorescens* PC24 was *Pseudomonas gessardii* CIP105469 (GenBank accession No. AFO74384).

3.2. Localisation of xylE, pheA and pchF genes of strains PC1, PC18 and PC24

The *xylE* gene probe of strain PC18 hybridised with the small plasmid replicon of this strain (Fig. 2A). Since we could not detect any plasmid DNA in strain PC1 and obtained a hybridisation signal at the position of chromosomal DNA, we hybridised the *xylE* gene probe of strain PC1 with *Sma*I restriction fragments of total DNA of strains PC1 and PC18 (Fig. 2B). In both cases we observed hybridisation, suggesting chromosomal location of the C23O gene in strain PC1 (Fig. 2B). The PMO of strain

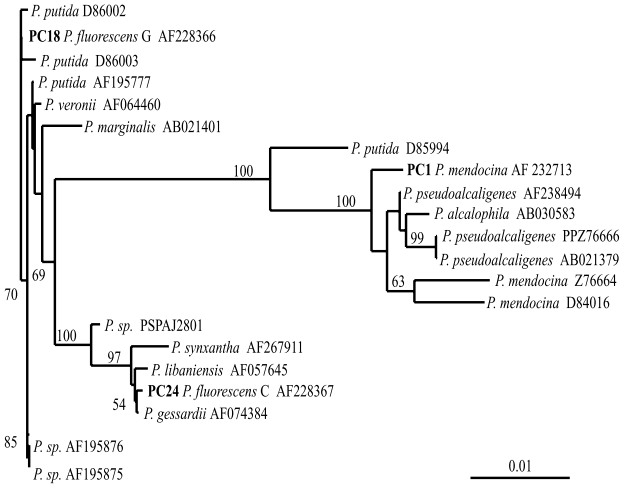
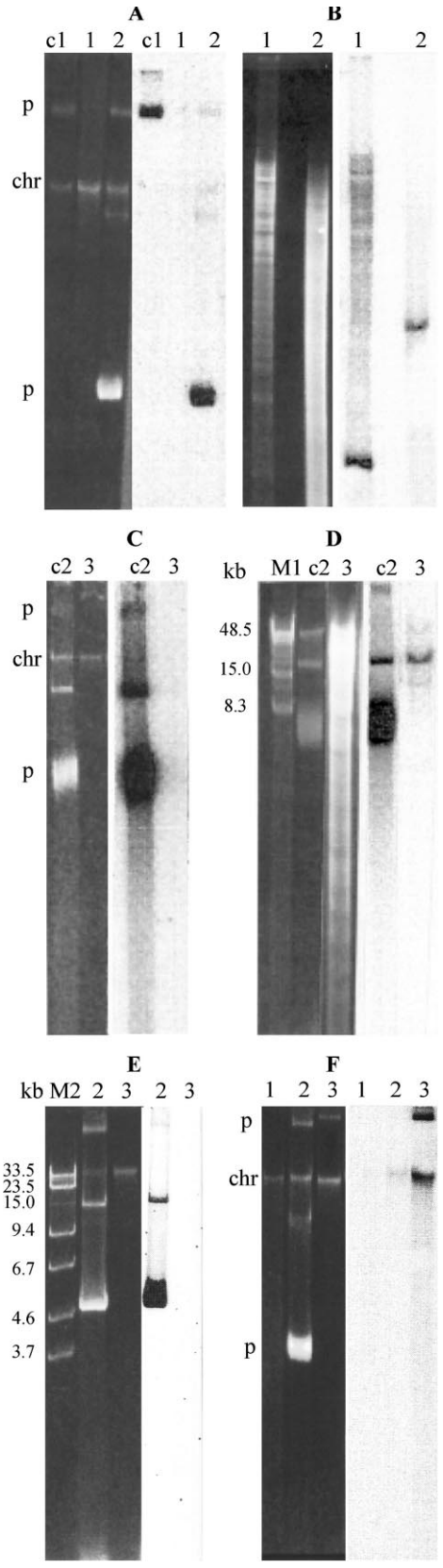


Fig. 1. Unrooted neighbour-joining tree, based on partial 16S rDNA sequence data, showing relationships of *P. mendocina* PC1, *P. fluorescens* G PC18, *P. fluorescens* C PC24 (in bold) and related species. Bootstrap values over 50% that supported the branching order, calculated by the neighbour-joining method from 100 replicates, are shown above the corresponding nodes. The scale bar represents 0.01 base substitutions per nucleotide.

PC24 was also chromosomally encoded. The *pheA* gene probe hybridised with an *Eco*RI fragment of total DNA of PC24, but not with plasmid DNA of this strain (Fig. 2C,D). The *pchF* gene-specific probes obtained from strains PC18 and PC24 yielded strong hybridisation with the plasmid DNA of the strain from which the probe was derived, while only a weak hybridisation signal was detected with plasmid DNA from the other strain (Fig. 2E,F). The size of PCR amplified fragments of the PCMH gene from strains PC18 and PC24 was the same (450 bp with PCMHF primers and about 150 bp with PCMHC primers, respectively). Therefore we compared the nucleotide sequences of the PCR products of the *pchF* gene from strains PC18 and PC24 and found them to be 89% identical. Deduced amino acid sequences of the two fragments showed 95% identity. Identities of amino acid sequences of *pchF* gene products from strains PC18 and PC24 with PchF protein from *P. putida* strain NCIMB 9866 [33] were 84% and 87%, respectively (Fig. 3).

3.3. Decomposition of phenol in combination with p-cresol and accumulation of p-hydroxybenzoate

Phenol and *p*-cresol were utilised simultaneously during the growth of *P. mendocina* PC1 and *P. fluorescens* C PC24 on the mixture of phenol and *p*-cresol (Fig. 4A,C). Simultaneous utilisation of these compounds can be explained by the absence of metabolic conflict: strain PC1 degraded the above-mentioned compounds via the *meta* pathway by C23O, and strain PC24 via the *ortho* pathway (phenol by C12O and *p*-cresol by PC34O). The key enzymes of degradative pathways were shown to be induced (Table 1). In contrast, during the growth of *P. fluorescens* G PC18 on the mixture of phenol and *p*-cresol, typical diauxic growth was observed with *p*-cresol being the preferred substrate and degraded first (Fig. 4B). Enzyme activities in cells collected before and after the intermediate plateau revealed high levels of PC34O and C23O activities in the first and second exponential growth phase, respectively (Table 1). The delay in decomposition of phenol can



→

Fig. 2. Agarose gel electrophoresis of plasmid (A,C,E,F) and digested genomic DNA (B,D) from *P. mendocina* strain PC1 (lane 1), *P. fluorescens* strains PC18 (lane 2) and PC24 (lane 3) in the left panels and corresponding Southern blot hybridisation in the right panels. Total DNA was digested with *Sma*I (B, lanes 1, 2) and *Eco*RI (D, lane 3). Hybridisations were performed with ^{32}P-labelled DNA using a 356-bp PCR amplification product of *xylE* genes [22] from strains PC18 (A) and PC1 (B), a 947-bp product of the *pheA* gene [22] from strain PC24 (C,D) and a 450-bp *pchF* gene product (see Section 2) from strains PC18 (E) and PC24 (F). The reference plasmids for the *xylE* and *pheA* genes were isolated from the strains *P. putida* E2 [23] (lane c1) and *P. putida* EST1026 (pEST1026) [24] (lane c2), respectively. Lambda mix (lane M1) and *Bsp*681/*Xho*I restriction fragments of λDNA (lane M2) were used as the DNA size standards. chr, chromosomal DNA; p, plasmid DNA.

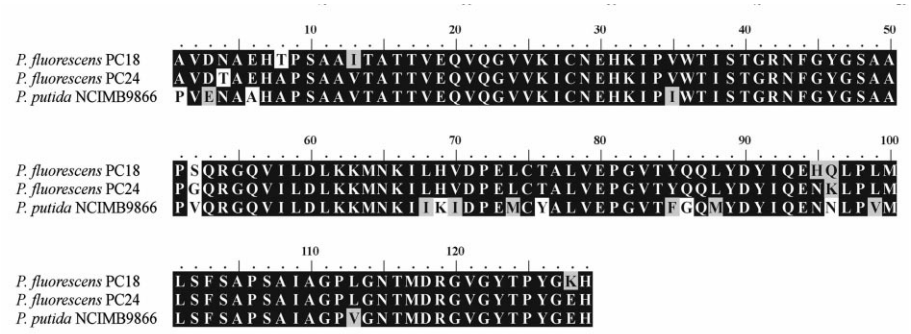


Fig. 3. Amino acid sequence alignment of PCMHs from the *P. fluorescens* strains PC18, PC24 and the type strain *P. putida* NCIMB 9866. Residues identical in at least two aligned sequences are in black boxes. Similar residues are in grey boxes.

be explained by limitations in the protocatechuate *ortho* pathway, because during degradation of *p*-cresol POB as an intermediate of this pathway was excreted and accumulated in the growth medium. Phenol degradation via the catechol *meta* pathway started only after decomposition of POB (Fig. 4B). Notably, during nondiauxic growth on the phenol and *p*-cresol mixture (strain PC24) we detected only very moderate accumulation of POB and *cis*,*cis*-muconate (CCM). High specific activities of POBH in the first exponential growth phase in strain PC18 showed that the accumulation of POB was not caused by a pathway bottleneck at the POBH step (Table 1).

3.4. Degradation of phenol and p-cresol in combination with POB

If the accumulation of POB is repressing the expression of the phenol *meta* pathway in a phenol and *p*-cresol mixture in strain PC18, then diauxie should occur also during the growth of this strain in the POB–phenol mixture. Indeed, in this case, diauxic growth was observed and degradation of phenol took place only after the consumption of POB (Fig. 5A). The behaviour of strain PC24 was different. It consumed POB (an intermediate of *p*-cresol degradation) and phenol simultaneously but CCM accumulated in the medium as an intermediate of phenol degradation (Fig. 5B). However, the formation of CCM (up to 9 mg l^{-1}) did not repress degradation of POB and phenol. The control cultivation experiments of strains PC18 and PC24 on POB–*p*-cresol mixtures indicated nondiauxic growth, as was expected (Fig. 5C,D). Thus, these substrates can be consumed simultaneously. However, actual data for strain PC18 showed that degradation of *p*-cresol was approx. 70% complete before degradation of POB began (Fig. 5C). We suppose that the preferential utilisation of *p*-cresol over POB can be apparent. Degra-

Table 1
Specific activities of key catabolic enzymes in crude extracts of *P. mendocina* PC1, *P. fluorescens* G PC18, *P. fluorescens* C PC24 and *P. putida* E2 grown on mixtures of phenolic compounds[a]

Strain	Substrates of growth	Growth phase	Spec. act. (nmol min^{-1} mg^{-1} of protein) of enzymes[b]				
			PMO	C12O	C23O	PC34O	POBH
PC1	PHE+*p*-CRE	i	< 1	16 ± 4	540 ± 44	27 ± 14	< 1
	PHE+BEN	ii	< 1	590 ± 62	9 ± 4	< 1	15 ± 5
		iii	< 1	699 ± 76	444 ± 29	< 1	13 ± 6
	p-CRE+BEN	ii	< 1	836 ± 70	< 1	< 1	14 ± 3
		iii	< 1	1058 ± 55	1242 ± 18	< 1	47 ± 7
PC18	PHE+*p*-CRE	ii	< 1	< 1	26 ± 13	654 ± 92	323 ± 24
		iii	< 1	< 1	645 ± 59	129 ± 32	153 ± 19
	PHE+BEN	ii	< 1	666 ± 77	296 ± 34	203 ± 38	262 ± 22
		iii	< 1	229 ± 41	611 ± 74	56 ± 7	155 ± 13
	p-CRE+BEN	ii	< 1	38 ± 16	< 1	394 ± 44	466 ± 36
		iii	< 1	1270 ± 112	81 ± 4	290 ± 23	286 ± 20
PC24	PHE+*p*-CRE	i	114 ± 26	1426 ± 102	< 1	278 ± 19	86 ± 9
	PHE+BEN	i	60 ± 18	1008 ± 143	< 1	205 ± 18	80 ± 7
E2	PHE+BEN	i	< 1	< 1	3890 ± 59	< 1	< 1
	PHE+*p*-CRE	i	< 1	< 1	1210 ± 33	< 1	< 1

[a]Samples from two substrate mixtures of phenol (PHE), *p*-cresol (*p*-CRE) and benzoate (BEN) were collected from mid-exponential phase (i) or in the case of diauxic growth from first (ii) and second mid-exponential phases (iii).

[b]Specific activity values of phenol monooxygenase (PMO), catechol 1,2-dioxygenase (C12O), catechol 2,3-dioxygenase (C23O), protocatechuate 3,4-dioxygenase (PC34O) and *p*-hydroxybenzoate hydroxylase (POBH) are means of three independent experiments ± S.E.M.

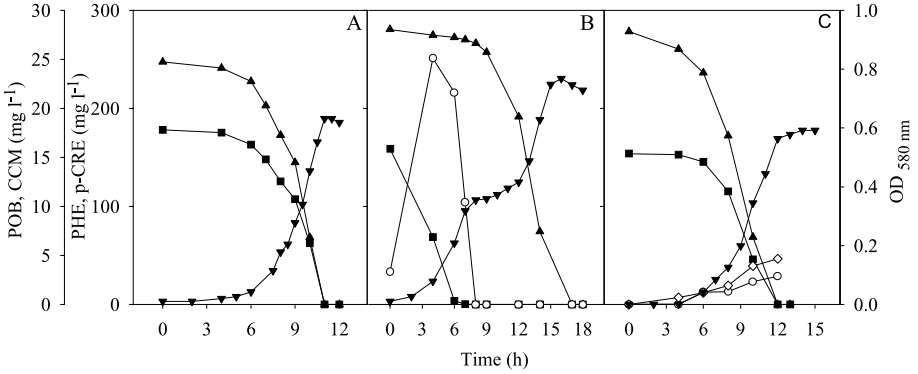


Fig. 4. Kinetics of growth, substrate consumption and accumulation of metabolites in strains PC1 (A), PC18 (B) and PC24 (C) cultivated on a mixture of phenol and *p*-cresol. Growth (▼) and concentrations of phenol (▲), *p*-cresol (■), *p*-hydroxybenzoate (○), *cis,cis*-muconate (◇).

dation of *p*-cresol yields POB that accumulates in the medium and contributes to the amount of POB in the medium.

3.5. Degradation of low concentrations of phenol and p-cresol in combination with POB by strain PC18

Under typical batch growth conditions diauxic or sequential utilisation of carbon substrates from mixtures is usual, while at low substrate concentrations the same substrates can be utilised simultaneously [38]. Therefore we decided to study the degradation of phenol, *p*-cresol and POB at concentrations 200–400 times lower than those used in previous experiments. As shown in Fig. 6A, only 10% of POB was utilised before phenol degradation began in strain PC18. It is obvious that, at very low substrate concentrations, simultaneous degradation occurred, whereas the antagonistic effect of POB on the degradation of phenol was significantly diminished. In contrast, POB and *p*-cresol were degraded from their mixture at the same time (Fig. 6B).

3.6. Phenol degradation in combination with benzoate and accumulation of catechol or cis,cis-muconate

To determine whether the catechol *ortho* pathway could be the limiting step of phenol degradation via the catechol *meta* pathway, we studied degradation of phenol in combination with benzoate. Benzoate degradation in strains PC1, PC18 and PC24 proceeds via the catechol *ortho* pathway by induction of C12O [22]. In the laboratory-constructed strain, *P. putida* AC783 E2, which lacks catechol and protocatechuate *ortho* pathway activities, phenol and benzoate were degraded through the catechol *meta* pathway by the induction of C23O (Table 1), enabling simultaneous utilisation of these two substrates from mixtures (Fig. 7B). No diauxie was observed during the growth of strain PC24 on the mixture of phenol–benzoate, and both

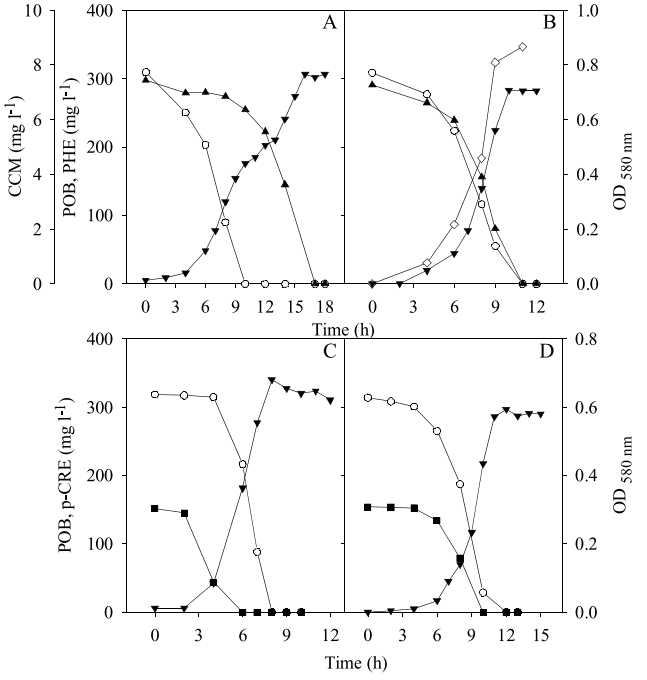


Fig. 5. Kinetics of growth, substrate degradation and accumulation of metabolites in strains PC18 (A,C) and PC24 (B,D) grown on mixtures of phenol–*p*-hydroxybenzoate (A,B) and *p*-cresol–*p*-hydroxybenzoate (C,D). Growth (▼) and concentrations of phenol (▲), *p*-cresol (■), *p*-hydroxybenzoate (○), *cis,cis*-muconate (◇).

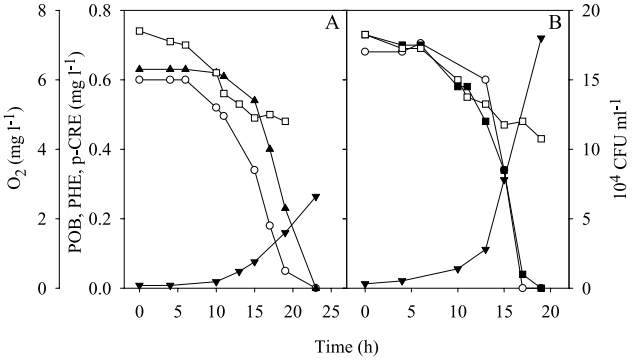


Fig. 6. Kinetics of growth at low concentrations of phenolic compounds by strain PC18 on mixtures of phenol–*p*-hydroxybenzoate (A) and *p*-cresol–*p*-hydroxybenzoate (B). Growth (▼) and concentrations of phenol (▲), *p*-cresol (■), *p*-hydroxybenzoate (○), oxygen (□).

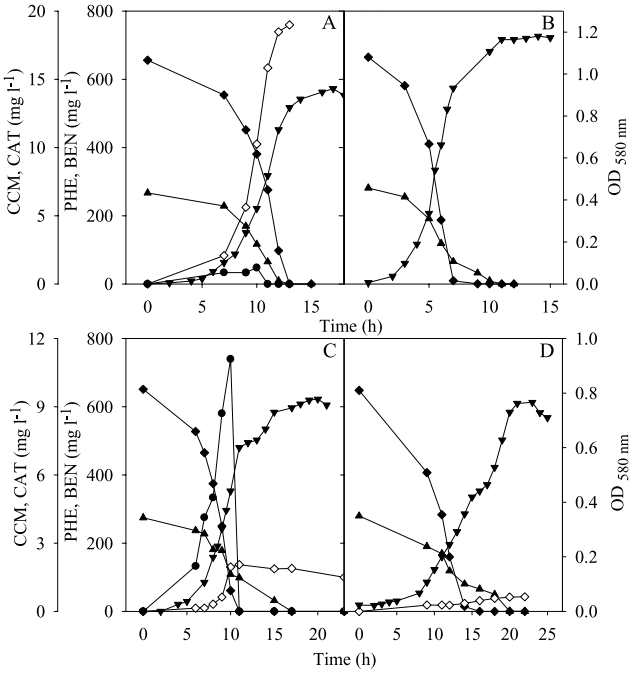


Fig. 7. Kinetics of growth, substrate degradation and accumulation of metabolites on a mixture of phenol–benzoate by strains PC24 (A), E2 (B), PC1 (C) and PC18 (D). Growth (▼) and concentrations of phenol (▲), benzoate (♦), *cis*,*cis*-muconate (◇), catechol (●).

substrates were catabolised simultaneously via the catechol *ortho* pathway by the induction of C12O (Fig. 7A and Table 1). Nevertheless, low reversible accumulation of catechol (1.2 mg l^{-1}) and high irreversible accumulation of CCM (19 mg l^{-1}) in the growth medium showed that the degradation of substrates was incomplete. On the other hand, strains PC1 (Fig. 7C) and PC18 (Fig. 7D) had typical diauxic growth on the phenol–benzoate mixture. According to the enzyme induction data, benzoate was used in the first exponential phase by C12O and phenol in the second exponential phase by C23O (Table 1). We suppose that in strain PC1 reversible accumulation of catechol (up to a concentration of 11 mg l^{-1}) from benzoate was the reason for diauxic growth. However, for strain PC18 there

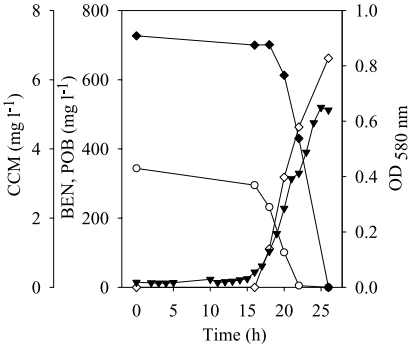


Fig. 8. Kinetics of growth, substrate degradation and accumulation of metabolites on the mixture benzoate–*p*-hydroxybenzoate by strain PC18. Growth (▼) and concentrations of benzoate (♦), *p*-hydroxybenzoate (○), *cis*,*cis*-muconate (◇).

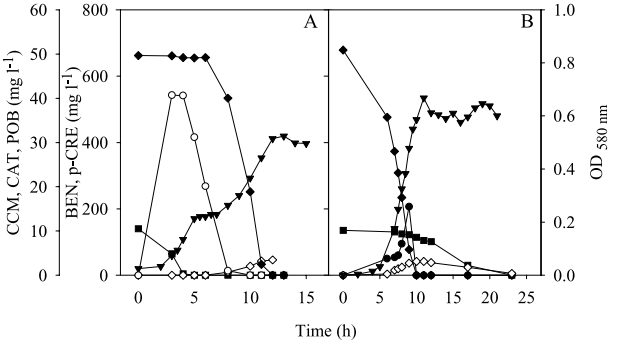


Fig. 9. Kinetics of growth, substrate degradation and accumulation of metabolites on a mixture of benzoate–*p*-cresol by strains PC18 (A) and PC1 (B). Growth (▼) and concentrations of benzoate (♦), *p*-cresol (■), *p*-hydroxybenzoate (○), catechol (●) and *cis*,*cis*-muconate (◇).

was no evidence that the diauxic growth on the phenol–benzoate mixture resulted from accumulation of catechol. We suggest that irreversible accumulation of CCM (0.6–20 mg l^{-1}) during growth of strains PC1, PC18 and PC24 on phenol–benzoate is the reason for inefficient decomposition of growth substrates.

3.7. Benzoate degradation in combination with POB and *p*-cresol

To assess the physiological significance of POB overflow to the catechol *ortho* pathway, we studied benzoate degradation in combination with POB and *p*-cresol (Figs. 8 and 9). During the growth of strain PC18 on the mixture of benzoate–POB, a long lag-phase was observed, but utilisation of benzoate started only slightly after POB (Fig. 8). Degradation of benzoate was accompanied by accumulation of CCM (up to 7 mg l^{-1}). During growth on the benzoate–*p*-cresol mixture strain PC18 preferred *p*-cresol to benzoate (Fig. 9A). In this case POB transiently accumulated in the medium up to 40 mg l^{-1} and benzoate consumption began only after the diauxie. The enzymes specific for benzoate catabolism showed a more pronounced increase in activity in the second growth phase (Table 1). These results show that POB is the key compound also triggering the repression of the catechol *ortho* pathway. During growth on the benzoate–*p*-cresol mixture the preferred usage of benzoate over *p*-cresol by strain PC1 was observed (Fig. 9B). The accumulation of catechol (up to 16 mg l^{-1}) repressed the catechol *meta* pathway and derepression of C23O activity in the second growth phase was observed (Table 1).

4. Discussion

We recently isolated three representative strains (*P. mendocina* PC1, *P. fluorescens* G PC18 and *P. fluorescens* C PC24) degrading phenol and *p*-cresol via the catechol *meta-meta*, catechol *meta*-protocatechuate *ortho* and cate-

chol *ortho*-protocatechuate *ortho* branches, respectively, from river water continuously polluted by phenolic compounds [22]. Thus, even under strong selective pressure, natural phenotypic and genotypic plasticity of *Pseudomonas* spp. was observed. The same conclusion was made for pseudomonads isolated from a biological treatment plant of phenol-polluted waters [39]. It is generally accepted that two strains with 16S rRNA gene sequence homology of about 97.5% belong to the same species [40]. Indeed, *P. fluorescens* biotypes G and C (strains PC18 and PC24) share 99% homology and are representatives of the same species. Comparison of these sequences with that of *P. mendocina* PC1 revealed only 96% similarity, being indicative of different species. The analysis of 16S rRNA gene sequences shows that these three strains are located in different monophyletic clusters of the genus *Pseudomonas* (Fig. 1). Additionally, the strains investigated differed in the localisation of genes for key catabolic enzymes (Fig. 2). In strain PC1 the C23O gene *xylE* was localised on the chromosome despite the fact that it is a TOL plasmid pWWO type gene [22]. The *pheA* gene has also been characterised previously as a plasmid-borne gene [41] but in strain PC24 it was located on the chromosome (Fig. 2C,D). On the other hand, the C23O gene *xylE* in strain PC18 and the PCMH genes *pchF* in strains PC18 and PC24 were located on plasmid DNA.

Understanding the catabolic regulation in multi-nutrient environments requires techniques that can detect the complex array of metabolites produced during degradation of carbon source mixtures [19]. In the present work we studied the accumulation of key catabolic intermediates during the consumption of *p*-cresol, phenol and benzoate in two-substrate cultivations of three different strains. The activities of the key enzymes of the catabolic pathways during each exponential growth phase in different substrate mixtures were also monitored. We found that if the substrates were degraded from mixtures only via the catechol *meta*, via the protocatechuate *ortho* and catechol *ortho* pathways, or only via the catechol *ortho* pathways, then both substrates were consumed simultaneously (Fig. 4A,C and 7A,B). In these cases no accumulation of intermediates was found, except during simultaneous degradation of both phenol and benzoate via the catechol *ortho* pathway by strain PC24, where irreversible accumulation of CCM (up to 19 mg l^{-1}) in the growth medium was detected (Fig. 7A). However, accumulation of high levels of CCM did not inhibit degradation of phenol in the presence of benzoate. Thus, no metabolic conflict was detected in the case of degradation of different substrates through the same catabolic pathway.

The most interesting type of metabolic regulation was revealed in strain PC18 during growth on a phenol–*p*-cresol mixture. In this strain both substrates induce the protocatechuate *ortho* pathway through induction of PCMH [22]. We assume that induction of this enzyme causes accumulation of the intermediate POB, which at high concentration represses the activity of the catechol *meta* pathway and thereby coordinates utilisation of these two incompatible metabolic substrates. In contrast, the activity of PCMH was not induced by phenol in strain PC24. Comparison of the 450-bp region of PCMH flavoprotein subunit genes of strains PC18 and PC24 shows that the sequences are functionally similar (95% identical and 98% similar at the protein level) but evolutionary more diverse (89% identical at the nucleotide level). Moreover, the PCMH flavoprotein subunit genes of strains PC18 and PC24 revealed strong evolutionary divergence from the same gene of *P. putida* strain NCIMB 9866 (only 81% identity at the nucleotide sequence level) [33]. We assume that the ability of phenol to induce the protocatechuate *ortho* pathway in strain PC18 is a strain specific genetic trait.

It is well known that *meta* and *ortho* pathways are alternative for metabolic reasons [42,11]. In the present study we show that if the two substrates are consumed via *meta* and *ortho* pathways, then sequential consumption of substrates (and diauxic growth) occurs and the catabolic pathway for decomposition of the growth substrate used second is repressed by reversible accumulation of the intermediate product of the first catabolic pathway. We suppose that the inability of strain PC18 to degrade phenol and *p*-cresol simultaneously might be caused by transient accumulation of POB (Fig. 4B), because induction of the second catabolic pathway (catechol *meta* pathway) occurs only after exhaustion of this protocatechuate *ortho* pathway intermediate. This hypothesis is supported by the finding that phenol consumption was prevented by POB added as a growth substrate in strain PC18 (Fig. 5A) but not in strain PC24 (Fig. 5B). The preferred consumption of protocatechuate via the *ortho* pathway in strain PC18 might be caused by the high level of induction of the protocatechuate *ortho* pathway enzymes by phenol, and a low level of induction of catechol pathway enzymes [22]. In strain PC24, in which phenol is degraded via the catechol *ortho* pathway, degradation of phenol and POB occurred simultaneously without inhibition by POB, because both branches of the *ortho* pathway were induced. In this case the catechol *ortho* pathway induction is supported by the presence of the *pheBA* operon, encoding both PMO and C12O [22].

Our results do not confirm repression by benzoate of the utilisation of POB in *P. putida* [8] and in *A. calcoaceticus*, [19] in which benzoate or its metabolic descendants were involved in inhibition of POB utilisation. In strain PC24, benzoate and POB are degraded simultaneously (data not shown) and in strain PC18 the degradation of benzoate was blocked by POB (Fig. 8). Moreover, our results indicate that POB inhibits not only the catechol *meta* pathway of phenol degradation, but also the catechol *ortho* pathway at the degradation of benzoate as a second growth substrate (Fig. 9A).

The mechanisms governing preferential benzoate con-

sumption via the catechol *ortho* pathway have not yet been elucidated. Transient accumulation of catechol in culture media during mixed substrate consumption has been explained by the high concentrations of benzoate used [17,14]. We found that sequential decomposition of both phenol and *p*-cresol via the catechol *meta* pathway from mixtures of two substrates might be the response to reversible accumulation of catechol from benzoate in strain PC1 (Fig. 7C and 9B). It is not likely that the second product of catechol *ortho* ring cleavage, *cis*,*cis*-muconate, inhibits the catechol *meta* pathway, as indicated for POB utilisation by *A. calcoaceticus* [19]. In our experiments even high external concentrations of CCM (up to 19 mg l^{-1}) did not inhibit degradation of phenol via the catechol *ortho* pathway (Fig. 7A).

We could show that at low concentrations of POB, the repression by this compound on the consumption of phenol was less expressed (Fig. 6A). The quantitative aspect of genetic and biochemical regulation of catabolic pathways is based on the assumption that, at very low substrate concentrations, the catabolic repression would be diminished [38]. This is in good agreement with our experimental results. Nevertheless, for further understanding of the mechanisms regulating catabolic pathways in two-substrate cultivations more detailed investigation at the molecular level is needed.

Acknowledgements

This research was supported by the Institute of Molecular and Cell Biology, University of Tartu, and by Estonian Science Foundation Grant 4344. We also thank Dr. T. Alamäe and Dr. A. Nurk for helpful discussions.

References

[1] Pieper, H.D. and Reineke, W. (2000) Engineering bacteria for bioremediation. Curr. Opin. Biotechnol. 11, 262–270.
[2] Jahnke, M., Lehmann, F., Schoebel, A. and Auling, G. (1993) Transposition of the TOL catabolic genes (Tn 4651) into the degradative plasmid pSAH of *Alcaligenes* sp. 0-1 ensures simultaneous mineralization of sulpho- and methyl-substituted aromatics. J. Gen. Microbiol. 139, 1959–1966.
[3] Duetz, W.A., Marques, S., Wind, B., Ramos, J.L. and van Andel, J.G. (1996) Catabolite repression of the toluene degradation pathway in *Pseudomonas putida* harboring pWW0 under various conditions of nutrient limitation in chemostat culture. Appl. Environ. Microbiol. 62, 601–606.
[4] Holtel, A., Marqués, S., Möhler, I., Jakubzik, U. and Timmis, K.N. (1994) Carbon source-dependent inhibition of *xyl* operon expression of the *Pseudomonas putida* TOL plasmid. J. Bacteriol. 176, 1773–1776.
[5] Müller, C., Petruschka, L., Cuypers, H., Burchhardt, G. and Herrmann, H. (1996) Carbon catabolite repression of phenol degradation in *Pseudomonas putida* is mediated by the inhibition of the activator protein PhlR. J. Bacteriol. 178, 2030–2036.
[6] Duetz, W.A., Marqués, S., de Jong, C., Ramos, J.L. and van Andel, J.G. (1994) Inducibility of the TOL catabolic pathway in *Pseudomonas putida* (pWW0) growing on succinate in continuous culture: evidence of carbon catabolite repression control. J. Bacteriol. 176, 2354–2361.
[7] Zylstra, G.J., Olsen, R.H. and Ballou, D.P. (1989) Cloning, expression, and regulation of the *Pseudomonas cepacia* protocatechuate 3,4-dioxygenase genes. J. Bacteriol. 171, 5907–5914.
[8] Nichols, N.N. and Harwood, C.S. (1995) Repression of 4-hydroxybenzoate transport and degradation by benzoate: a new layer of regulatory control in the *Pseudomonas putida* β-ketoadipate pathway. J. Bacteriol. 177, 7033–7040.
[9] Hester, K.L., Lehman, J., Najar, F., Song, L., Roe, B.A., MacGregor, C.H., Hager, P.W., Phibbs Jr., P.V. and Sokatch, J.R. (2000) Crc is involved in catabolite repression control of the *bkd* operons of *Pseudomonas putida* and *Pseudomonas aeruginosa*. J. Bacteriol. 182, 1144–1149.
[10] Ornston, L.N. and Stanier, R.Y. (1966) The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. J. Biol. Chem. 241, 3776–3786.
[11] Schmidt, E. (1987) Response of a chlorophenol degrading mixed culture to changing loads of phenol, chlorophenol and cresols. Appl. Microbiol. Biotechnol. 27, 94–99.
[12] Keat, M. and Hopper, D.J. (1978) *p*-Cresol and 3,5-xylenol methylhydroxylases in *Pseudomonas putida* N.C.I.B. 9869. Biochem. J. 175, 649–658.
[13] Bossert, I.D., Whited, G., Gibson, D.T. and Young, L.Y. (1989) Anaerobic oxidation of *p*-cresol mediated by a partially purified methylhydroxylase from a denitrifying bacterium. J. Bacteriol. 171, 2956–2962.
[14] Ampe, F. and Lindley, N.D. (1996) Flux limitations in the *ortho* pathway of benzoate degradation of *Alcaligenes eutrophus*: metabolite overflow and induction of the *meta* pathway at high substrate concentrations. Microbiology 142, 1807–1817.
[15] Knackmuss, H.-J. and Hellweg, M. (1978) Utilization and cooxidation of chlorinated phenols by *Pseudomonas* sp. B13. Arch. Microbiol. 117, 1–7.
[16] Ampe, F., Léonard, D. and Lindley, N.D. (1998) Repression of phenol catabolism by organic acids in *Ralstonia eutropha*. Appl. Environ. Microbiol. 64, 1–6.
[17] Ampe, F. and Lindley, N.D. (1995) Acetate utilization is inhibited by benzoate in *Alcaligenes eutrophus*: evidence for transcriptional control of the expression of *acoE* coding for acetyl coenzyme A synthetase. J. Bacteriol. 177, 5826–5833.
[18] Cowles, C.E., Nichols, N.N. and Harwood, C.S. (2000) BenR, XylS homologue, regulates three different pathways of aromatic acid degradation in *Pseudomonas putida*. J. Bacteriol. 182, 6339–6346.
[19] Gaines III, G.L., Smith, L. and Neidle, E.L. (1996) Novel nuclear magnetic resonance spectroscopy methods demonstrate preferential carbon source utilization by *Acinetobacter calcoaceticus*. J. Bacteriol. 178, 6833–6841.
[20] Copley, S.D. (2000) Evolution of a metabolic pathway for degradation of a toxic xenobiotic: the patchwork approach. Trends Biochem. Sci. 25, 261–265.
[21] van der Meer, J.R., de Vos, W.M., Harayama, S. and Zehnder, A.J.B. (1992) Molecular mechanisms of genetic adaptation to xenobiotic compounds. Microbiol. Rev. 56, 677–694.
[22] Heinaru, E., Truu, J., Stottmeister, U. and Heinaru, A. (2000) Three types of phenol and *p*-cresol catabolism in phenol- and *p*-cresol-degrading bacteria isolated from river water continuously polluted with phenolic compounds. FEMS Microbiol. Ecol. 31, 195–205.
[23] Wand, H., Laht, M., Peters, M., Becker, P.M., Stottmeister, U. and Heinaru, A. (1997) Monitoring of biodegradative *Pseudomonas putida* strains in aquatic environments using molecular techniques. Microb. Ecol. 33, 124–133.
[24] Kivisaar, M., Hõrak, R., Kasak, L., Heinaru, A. and Habicht, J. (1990) Selection of independent plasmids determining phenol degradation in *Pseudomonas putida* and the cloning and expression of

genes encoding phenol monooxygenase and catechol 1,2-dioxygenase. Plasmid 24, 25–36.
[25] Beadle, T.A. and Smith, A.R.V. (1982) The purification and properties of 2,4-dichlorophenol hydroxylase from a strain of *Acinetobacter species*. Eur. J. Biochem. 123, 323–332.
[26] Feist, C.F. and Hegeman, G.D. (1969) Phenol and benzoate metabolism by *Pseudomonas putida*: regulation of tangential pathway. J. Bacteriol. 100, 869–877.
[27] Hegeman, G.D. (1966) Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*. 1. Synthesis of enzymes by the wild type. J. Bacteriol. 91, 1140–1154.
[28] Krooneman, J., Wieringa, E.B.A., Moore, E.R.B., Gerritse, J., Prins, R.A. and Gottschal, J.C. (1996) Isolation of *Alcaligenes* sp. strain L6 at low oxygen concentrations and degradation of 3-chlorobenzoate via a pathway not involving (chloro)catechols. Appl. Environ. Microbiol. 62, 2427–2434.
[29] Bradford, M.M. (1976) A rapid sensitive method for the quantification of microgram quantities of protein-due binding. Anal. Biochem. 72, 248–254.
[30] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[31] Connors, M.A. and Barnsley, E.A. (1982) Naphthalene plasmids in pseudomonads. J. Bacteriol. 149, 1096–1101.
[32] Vedler, E., Kõiv, V. and Heinaru, A. (2000) Analysis of the 2,4-dichlorophenoxyacetic acid-degradative plasmid pEST4011 of *Achromobacter xylosoxidans* subsp. *denitrificans* strain EST4002. Gene 255, 281–288.
[33] Kim, J., Fuller, J.H., Cecchini, G. and McIntire, W. (1994) Cloning, sequencing, and expression of the structural genes for the cytochrome and flavoprotein subunits of *p*-cresol methylhydroxylase from two strains of *Pseudomonas putida*. J. Bacteriol. 176, 6349–6361.
[34] Maidak, B.L., Olsen, G.J., Larsen, N., Overbeek, R., McCaughey, M.J. and Woese, C.R. (1997) The RDP (Ribosomal Database Project). Nucleic Acids Res. 25, 109–111.
[35] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.
[36] Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
[37] Felsenstein, J. (1989) PHYLIP-phylogeny inference package (v3.5). Cladistics 5, 164–166.
[38] Kovárová-Kovar, K. and Egli, T. (1998) Growth kinetics of suspended microbial cells: from single-substrate-controlled growth to mixed-substrate kinetics. Microbiol. Mol. Biol. Rev. 62, 646–666.
[39] Whiteley, A.S., Wiles, S., Lilley, A.K., Philp, J. and Bailey, M.J. (2001) Ecological and physiological analyses of pseudomonad species within a phenol remediation system. J. Microbiol. Methods 44, 79–88.
[40] Stackebrandt, E. and Goebel, B.M. (1994) Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44, 846–849.
[41] Nurk, A., Kasak, L. and Kivisaar, M. (1991) Sequence of the gene (*phe*A) encoding phenol monooxygenase from *Pseudomonas* sp. EST1001 expression in *Escherichia coli* and *Pseudomonas putida*. Gene 102, 13–18.
[42] Klecka, G.M. and Gibson, D.T. (1981) Inhibition of catechol 2,3-dioxygenase from *Pseudomonas putida* by 3-chlorocatechol. Appl. Environ. Microbiol. 41, 1159–1165.