RESEARCH ARTICLE

Diversity of the transcriptional regulation of the *pch* gene cluster in two indigenous *p*-cresol-degradative strains of *Pseudomonas fluorescens*

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Abstract

p-Cresol methylhydroxylase (PCMH), a key enzyme responsible for the catabolism of *p*-cresol via the protocatechuate *ortho* pathway, was used as a tool to characterize catabolic differences between phenol- and *p*-cresol-degrading *Pseudomonas fluorescens* strains PC18 and PC24. Although both strains catabolize *p*-cresol using PCMH, different whole-cell kinetic parameters for this compound were revealed. Affinity for the substrate and the specific growth rate were higher in PC18, whereas maximum *p*-cresol tolerance was higher in PC24. In addition, PCMH of strain PC18 was induced during growth on phenol. In both strains, the *pchACXF* operon, which encodes *p*-hydroxybenzaldehyde dehydrogenase and PCMH, was sequenced. Transcriptional regulation of these operons by PchR, a putative σ^{54}-dependent regulator, was shown. Although the promoters of these operons resembled σ^{54}-controlled promoters, they differed from the consensus sequence by having T instead of C at position −12. Complementation assays confirmed that the amino acid sequence differences of the PchR regulators between the two strains studied led to different effector-binding capabilities of these proteins: (1) phenol was a more efficient effector for PchR of PC18 than *p*-cresol, (2) phenol did not activate the regulator of PC24, and (3) both regulators responded similarly to *p*-cresol.

Introduction

Methylphenols (cresols) are toxic compounds for living organisms. Cresols are produced in large amounts as constituents of resins, solvents, disinfectants, and wood-preserving chemicals in petrochemical processes, but they are also products of anaerobic tyrosine fermentation via *p*-hydroxy phenylacetate (Yu *et al.*, 2006). Fortunately, these compounds are degraded quite easily in nature both by aerobic (Hopper, 1976) and by anaerobic bacteria (Bossert & Young, 1986).

Two different catabolic routes have been described for *p*-cresol (4-methylphenol). In one of the pathways, a hydroxyl group is added to *p*-cresol and the resulting 4-methylcatechol is then cleaved by catechol *meta* pathway enzymes (Bayly *et al.*, 1966). The first enzyme that degrades *p*-cresol via the alternative route, the protocatechuate branch of the *ortho* pathway (also termed as the β-ketoadipate pathway), is *p*-cresol methylhydroxylase (PCMH, EC 1.17.99.1) (Hopper, 1976). This enzyme consists of two subunits to form an $\alpha_2\beta_2$ complex: the α subunits contain an active site FAD covalently linked to a tyrosine residue, whereas the β subunit is a *c*-type cytochrome (McIntire *et al.*, 1981, 1985). The natural electron acceptor for this periplasmic enzyme (Hopper *et al.*, 1985) is azurin (Causer *et al.*, 1984).

PCMH converts *p*-cresol to *p*-hydroxybenzyl alcohol and later to *p*-hydroxybenzaldehyde (Hopper, 1976; Cronin *et al.*, 1999; Cunane *et al.*, 2000), which is subsequently oxidized to *p*-hydroxybenzoate (POB) by *p*-hydroxybenzaldehyde dehydrogenase (Fig. 1). The formation of protocatechuate from POB is catalyzed by POB hydroxylase.

Despite studies showing that several *Pseudomonas* sp. and other microorganisms possess PCMH (Hopper, 1983; O'Reilly & Crawford, 1989; Lovley & Lonergan, 1990; Hopper *et al.*, 1991; Rudolphi *et al.*, 1991; Wright & Olsen, 1994; Heinaru *et al.*, 2000; Peters *et al.*, 2007), the genetics of the corresponding metabolic pathways has been studied in sufficient detail only in three *Pseudomonas* sp. strains: *Pseudomonas putida* NCIMB 9866, *P. putida* NCIMB 9869

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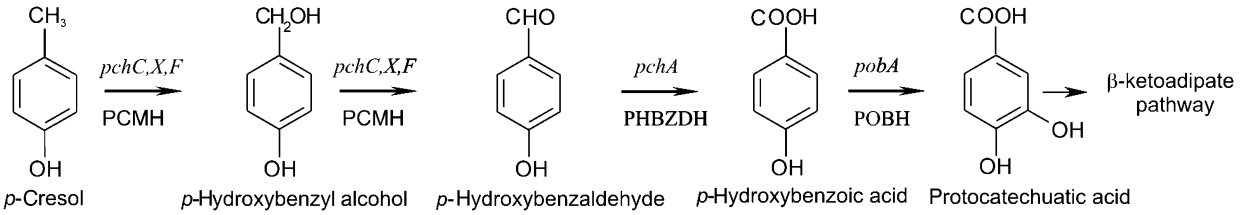

Fig. 1. *p*-Cresol biodegradation pathway via the prothocatechuate *ortho* pathway. PCMH, *p*-cresol methylhydroxylase; PHBZDH, *p*-hydroxybenzaldehyde dehydrogenase; POBH, *p*-hydroxybenzoate hydroxylase; *pchACXF*, *p*-cresol utilization genes; and *pobA*, codes for *p*-hydroxybenzoate hydroxylase.

(Kim *et al.*, 1994), and *Pseudomonas mendocina* KR1 (Wright & Olsen, 1994).

The genes encoding PCMH and *p*-hydroxybenzaldehyde dehydrogenase are organized as an operon (*pchACXF* in *P. putida* NCIMB 9866 and *pcuCAXB* in *P. mendocina* KR1), with *pchA*/*pcuC* encoding the second and *pchCF*/*pcuAB* encoding the first enzyme of the *p*-cresol metabolic pathway (Burlage *et al.*, 1989; Wright & Olsen, 1994). The gene designated as *pchX*/*pcuX* encodes a protein of unknown function (Wright & Olsen, 1994; Cronin *et al.*, 1999). The *pcuR* gene, transcribed divergently from the *pcuCAXB* operon, encodes the transcriptional regulator of this operon (Ramos-González *et al.*, 2002).

The best-characterized PCMH is the plasmid-encoded $PCMH_{69A}$ from *P. putida* NCIMB 9869, which is expressed when the organism is grown on *p*-cresol, 3,5-xylenol (i.e. 3,5-dimethylphenol), glutamate, or succinate as the carbon source. The chromosome-encoded $PCMH_{69B}$ genes of *P. putida* NCIMB 9869 are induced only when the organism is grown on *p*-cresol (Kim *et al.*, 1994). In *P. mendocina* KR1, these enzymes are involved in *p*-cresol metabolism; however, only this substrate was used as an inducer in this case (Wright & Olsen, 1994) (Fig. 1).

In this study, *Pseudomonas fluorescens* strains PC18 and PC24 were isolated from river water continuously polluted by phenolic oil shale semi-coke leachate, which contains mainly phenol and *p*-cresol as pollutants. Strains PC18 and PC24 are representatives of different catabolic types – they degrade *p*-cresol via the protocatechuate *ortho* pathway and phenol via the *meta* and *ortho* pathways, respectively (Heinaru *et al.*, 2000). It has been shown that when cultivated on a mixture of phenol and *p*-cresol, simultaneous degradation of these compounds occurs in PC24 and sequential degradation occurs in PC18 with *p*-cresol as the preferred substrate (Heinaru *et al.*, 2001). In this study, it was hypothesized that the inability of strain PC18 to degrade phenol and *p*-cresol simultaneously was caused by the transient accumulation of POB that represses the expression of catechol *meta* pathway enzymes, and PCMH activity was induced by phenol in strain PC18. The goal of the current investigation was to clarify the genetic background of the above-mentioned features of *p*-cresol catabolism in strains PC18 and PC24 by characterizing the *pch* operons, their transcriptional regulation, and enzyme induction.

Materials and methods

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are described in Table 1. Pure cultures were stored in 30% glycerol at −70 °C. The *Pseudomonas* strains were grown at 30 °C in liquid medium and on agar plates containing minimal medium with M9 salts (Adams, 1959) and trace elements (Bauchop & Elsden, 1960) supplemented with either phenol (2.5 mM) or *p*-cresol (1.3 mM). *Escherichia coli* DH5α harboring pTZ57R/T- or $pPR9TT_B$-based plasmids was grown at 37 °C on Luria–Bertani (LB) medium with either ampicillin (15 μg mL^{-1}) or chloramphenicol (20 μg mL^{-1}), respectively.

Growth

Batch cultivation of cells was performed in 150-mL Erlenmeyer flasks containing 50 mL of minimal medium supplemented with 1.3 mM *p*-cresol at 30 °C on a rotary shaker. Growth was followed spectrophotometrically at 580 nm. The specific growth rate (μ_{max}) of cultures was calculated using the Richards model (Dalgaard & Koutsoumanis, 2001).

Kinetic constants of *p*-cresol-oxygenating activity

The *p*-cresol-oxygenating activity of strains PC18 and PC24 pregrown on *p*-cresol was determined using a Clark-type oxygen electrode as described by Viggor *et al.* (2008). The apparent kinetic constants, half saturation constant (K_s), and maximum specific activity (V_{max}), were determined using a nonlinear regression method according to the Michaelis–Menten equation (SIGMAPLOT 2001, SYSTAT).

A respiratory system OxiTop® Control sensor (WTW, Germany) was used to determine the maximum *p*-cresol inhibitory concentration at which no oxygen consumption by bacteria is observed. The tests were performed in 250-mL bottles containing 100 mL of minimal medium supplemented

Table 1. Bacterial strains and plasmids used for this study

Strain or plasmid	Genotype or construction	Source or reference
P. fluorescens		
PC18 biotype B	Wild-type strain, Phe^+, $pCre^+$	Heinaru *et al*. (2001)
PC24 biotype C	Wild-type strain, Phe^+, $pCre^+$	Merimaa *et al*. (2006)
PC18pchR$^-$	Native *pchR* of strain PC18 knockout derivative with Km^r	This study
E. coli		
DH5α	*supE44 ΔlacU169* (φ*80 lacZ ΔM15*) *recA1 endA1 hsdR17 thi-1 gyrA96 relA1*	Invitrogen
HB101	*subE44 subF58 hsdS3* ($r_B^- m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	Boyer & Roulland-Dussoix (1969)
CC118λpir	Δ(*ara-leu*) *araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE* (*Am*) *recA1 λpir* phage lysogen	Herrero *et al*. (1990)
Plasmids		
pTZ57R/T	Cloning vector (Ap^r)	MBI Fermentas
pUTmini-Tn*5* Km2	Delivery plasmid for mini-Tn*5* Km2 (Ap^r Km^r)	De Lorenzo *et al*. (1990)
pGP704 L	Delivery plasmid for homologous recombination (Ap^r)	Pavel *et al*. (1994)
pRK2013	Helper plasmid for conjugal transfer of pGP704 L (Km^r)	Figurski & Helinski (1979)
$pPR9TT_B$	Low-copy-number *lacZ*-based promoter probe plasmid pPR9TT, derivative without the *lacZ* gene; Cm^r, Ap^r	Kivistik *et al*. (2006)
PC18R/pTZ57R	pTZ57R/T containing the PCR-amplified *pchR* gene of strain PC18	This study
pTZ57RΔ18pchR::km	*pchR* of strain PC18 in pTZ57R/T is interrupted with Km^r gene from pUTmini-Tn*5* Km2 by replacing Bsp1407I- and BglII-generated fragment from *pchR* by Km^r gene	This study
pGP704Δ18pchR::km	pGP704 L with SphI–SacI fragment of Δ18*pchR*::*km* from pTZ57RΔ18pchR::km in the vector plasmid opened with the same restrictases	This study
pTZ57R/T-18RBS	pTZ57R/T containing the PCR-amplified *pchR* gene and the upstream promoter–operator area of strain PC18	This study
pTZ57R/T-24RBS	pTZ57R/T containing the PCR-amplified *pchR* gene and the upstream promoter–operator area of strain PC24	This study
$pPR9TT_B$-18RBS	$pPR9TT_B$ with HindIII–XbaI fragment of 18RBS from pTZ57R/T-18RBS in the vector plasmid opened with the same restrictases	This study
$pPR9TT_B$-18RBS	$pPR9TT_B$ with HindIII–XbaI fragment of 24RBS from pTZ57R/T-24RBS in the vector plasmid opened with the same restrictases	This study
Δ$pPR9TT_B$-18RBS::km	18RBS in $pPR9TT_B$ is interrupted with the Km^r gene from pUTmini-Tn*5* Km2 by replacing Bsp1407I- and XbaI-generated fragment from *pchR* by the Km^r gene	This study

Km^r, resistance to kanamycin; Phe^+, $pCre^+$, the ability to degrade phenol and *p*-cresol, respectively.

with different concentrations (0.2–6.5 mM) of *p*-cresol at 20 °C for up to 5 days. The media in bottles were inoculated with bacteria pregrown on 1.3 mM *p*-cresol at an initial cell concentration of 10^6 CFU mL^{-1}. Oxygen consumption was calculated on the basis of the pressure decline in the bottles following CO_2 trapping by the absorbent (soda lime, Fluka). Basal respiration of strains under the same conditions, but without any carbon source, was used as a control. The specific oxygen-consumption rate (V) was calculated from semi-logarithmic consumption curves using linear regression. The Luong equation was used to describe the specific oxygen-consumption rate inhibition by *p*-cresol:

$$V = \frac{V_{\max} S}{K_s + S}\left(1 - \frac{S}{S_m}\right)^n$$

where V_{max} is the maximum specific oxygen-consumption rate, S is the initial concentration of *p*-cresol, K_s is the half-saturation constant, S_m is the maximum *p*-cresol inhibitory concentration at which no oxygen consumption was observed, and n is a power term, whose value determines the shape of the curve as S approaches S_m (in our calculations, it was set to be equal to 1).

DNA sequencing and analysis

Nucleotide sequencing was carried out on a 3730*xl* DNA Analyzer (Applied Biosystems) using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and protocols provided by the manufacturer. The GenBank database search was conducted using BLAST programs. The PC18 and PC24 nucleotide sequences obtained were aligned with those of *P. putida* NCIMB 9866 and *P. mendocina* KR1, and assembled according to these alignments using BIOEDIT version 7.0.5.3 (Hall, 1999). CLUSTAL W version 1.83 was used for sequence alignments and phylogenetic analysis (Thompson *et al*., 1994).

The nucleotide sequences of the *pch* operons of strains PC18 and PC24 were deposited in GenBank under the accession numbers GQ131728 and GQ131727, respectively.

Localization of the transcriptional initiation site of *pchACXF* operons

The transcriptional initiation sites of the *pch* operons of the two studied strains were established through 5′ rapid amplification of cDNA ends (RACE), essentially as described by Sambrook & Russell (2001). RNA was isolated from *P. fluorescens* strains PC18 and PC24 grown on *p*-cresol. Total RNA was extracted from exponential-phase cultures using the Nucleospin® RNA II extraction kit (Macherey-Nagel), according to the manufacturer's instructions. DNAse treatments were performed using total RNA of the strains. The first-strand cDNA was synthesized from total RNA with the *pch*-specific primer RA1 (5′-GCGTGATCTGCAGCAAGC-3′). Free nucleotides and primers were removed by twice precipitating cDNA in 2.5 M ammonium acetate with three volumes of 95% ethyl alcohol. cDNA was then dissolved in double-distilled water to a final volume of 20 μL. cDNA (13.5 μL) was used for poly(dG) tailing with terminal deoxyribonucleotidyl transferase, as recommended by the manufacturer (MBI Fermentas). Free nucleotides and primers were then removed from tailed DNA as described above, the tailed DNA was then dissolved in double-distilled water to a final volume of 30 μL, and 4 μL of it was used for PCR amplification with a poly(dC) primer (C-anchor) (5′-GGCCACGCGTCGACTAGTACC$_{15}$D 3′), the *pch*-specific primer RA2 (5′-CAGTTCAAGGTTTT GATAGGC-3′), and primer Arb2 (5′-GGCCACGCGTCGAC TAGTAC-3′) (Caetano-Anollés, 1993). The primers Arb1 (5′-GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT-3′) and Arb2 (Caetano-Anollés, 1993) were also used to analyze the ends of the genes *pchR* and *pchF*. The PCR products were purified using the Invisorb Fragment CleanUp kit (Invitek), according to the instructions provided. The PCR products were ligated into pTZ57R/T using the InsT/Aclone™ PCR Product Cloning Kit (MBI Fermentas) and transformed into *E. coli* DH5α competent cells (Inoue *et al.*, 1990). The *E. coli* cells were cultured on LB medium at 37 °C containing 15 μg mL^{-1} ampicillin, 48 μg mL^{-1} isopropyl-β-D-thiogalactopyranoside, and 80 μg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Colonies were analyzed by PCR using M13/pUC forward and reverse primers, and then the amplified PCR products were sequenced.

Construction of plasmids and strains

To disrupt the *pchR* gene in *P. fluorescens* strain PC18, the coding region of *pchR* was PCR amplified from genomic DNA of the studied strains with oligonucleotides pchR4 (5′-GYCYTAGCTTCCCTTGAGGC-3′) and pchR5 (5′-GTTT WAWCTGTGCGGTTATCG-3′). The *pchR*-containing PCR product was cloned into pTZ57R/T, resulting in PC18R/pTZ57R. The central region of *pchR* in PC18R/pTZ57R was excised with Bsp1407I and BglII and replaced with the Km^r gene. A fragment of approximately 1 kb of the *pchR* gene was deleted. The Km^r gene was amplified by PCR from the plasmid pUTmini-Tn5 Km2 using the primer KmSac (5′-CAGGAGCTCGTTCGATTTATTCAACAAAGCC-3′) (Hõrak *et al.*, 2004). The Ecl136II-cleaved DNA fragment containing the Km^r gene was inserted into the cleaved *pchR* gene of strain PC18. The Bsp1407I and BglII ends were blunt ended before ligation. The resulting Δ18pchR::km sequence from pTZ57RΔ18pchR::km was excised and inserted as the SphI–SacI fragment plasmid pGP704. The resulting plasmid pGP704Δ18pchR::km was conjugatively transferred from *E. coli* CC118λ pir (Herrero *et al.*, 1990) into *P. fluorescens* strain PC18 using the helper plasmid pRK2013 (Figurski & Helinski, 1979). Strain PC18*pchR*$^-$ was verified by PCR analysis using the primers KmOc (5′-TCGAGCAAGACG TTTCCC-3′) (Saumaa *et al.*, 2006) and pchR4.

A 2.4-kb DNA region containing the entire *pchR* gene and the upstream promoter–operator area was amplified in both strains using the primers pchR4 and pchRBS (5′-CTCTT ATTGGWGCGAAAGGGCATC-3′). The resulting fragment was inserted into the vector pTZ57R/T using the InsTAclone™ PCR Cloning Kit (MBI Fermentas). After digestion of the obtained constructs pTZ57R/T-18RBS and pTZ57R/T-24RBS with HindIII and XbaI, the fragment containing *pchR* with its own promoter–operator area was inserted into the corresponding sites of pPR9TT$_B$. After verification of the constructs by digestion with HindIII and XbaI, pPR9TT$_B$-18RBS was electrotransformed into strain PC24 and the transformants were selected on LB medium with carbenicillin (5000 μg mL^{-1}).

pPR9TT$_B$-24RBS was electrotransformed into PC18*pchR*$^-$. Transformants were selected on LB medium with kanamycin (50 μg mL^{-1}) and carbenicillin (5000 μg mL^{-1}), and verified by PCR analysis using the primers Km4 (5′-AATTGGTTGTA ACACTGGCAGA-3′) and pchRBS.

Mutant ΔpPR9TT$_B$-18RBS::km was constructed by restriction of *pchR* with Bsp14017 and XbaI, deleting an approximately 1500-bp fragment from *pchR*. The Bsp1407 and XbaI ends were blunt ended before the ligation and transformed into *E. coli* DH5α competent cells. Transformants were selected on LB medium with kanamycin (50 μg mL^{-1}) and verified by PCR analysis using the primers Km4 and pchRBS, and KmOc and pchRBS. Construct ΔpPR9TT$_B$-18RBS::km was electrotransformed into the wild type strain PC24, the transformants were selected on LB medium with kanamycin (50 μg mL^{-1}) and carbenicillin (5000 μg mL^{-1}), and verified by PCR analysis.

Enzyme activity assay

Cultures of *P. fluorescens* PC18 and PC24 were grown to the midexponential growth phase on minimal M9 medium supplemented with 0.2% casamino acid (CAA) and either 1.3 mM *p*-cresol or 2.5 mM phenol. Crude extracts were

prepared from cells twice washed with cold K_2HPO_4–KH_2PO_4 buffer (100 mM; pH 7.5), resuspended in the same buffer, and sonicated. Unbroken cells and cell debris were removed by centrifugation at 12 000 ***g*** for 25 min at 4 °C. PCMH activity was measured using a modified procedure of Bossert *et al.* (1989). A spectrophotometric 2,6-dichlorophenol-indophenol (DCPIP)/phenazine methosulfate (PMS) assay for PCMH activity was performed at 600 nm. Reaction mixtures contained the following: 1.1 μmol of PMS, 100 nmol of DCPIP, 1.2 μmol of *p*-cresol, cell-free extract, and 100 mM phosphate buffer (pH 7.5) to a final volume of 1.0 mL. The reaction was started by the addition of substrate and reduction of DCPIP was measured ($\varepsilon = 21\ cm^2\ \mu mol^{-1}$). Protein concentrations were measured using the Bradford method (Bradford, 1976) with bovine serum albumin as the standard.

Results and discussion

Whole-cell kinetic parameters of *p*-cresol oxygenation by PC18 and PC24

PCMH, as the first enzyme in the *p*-cresol-degrading pathway via the protocatechuate branch, determines the whole-cell kinetics of *p*-cresol-oxygenating activity. The specific growth rate, μ_{max}, was determined using the Richards model from absorbance values of cultures during batch cultivation experiments. The μ_{max} values obtained for strains PC18 and PC24 were 1.12 and 0.71 h^{-1}, respectively. Figure 2 shows that in addition to almost twofold higher μ_{max} values of PC18 for *p*-cresol growth, the length of the lag phase was also about threefold shorter compared with PC24.

The apparent half-saturation constant values for *p*-cresol-oxygenating activity (K_s) were calculated from data obtained using a Clark-type oxygen electrode by measuring the oxygen consumption rate dependence of strains PC18 and PC24 on the *p*-cresol concentration (Fig. 3a). The K_s values for strain PC18 (3.8 μM) were almost fivefold lower compared with strain PC24 (17.3 μM). A low K_s value for PC18 reflects a high affinity of the strain for *p*-cresol, which is in agreement with the values of *p*-cresol maximum inhibitory concentration at which no oxygen consumption was observed, (S_m), determined from respiration measurements. Namely, S_m of strain PC18 was lower than that of strain PC24 (Fig. 3b). These results are in accordance with the chemical composition of the habitat of the strains – PC24 was isolated from a ditch surrounding the oil shale semi-coke mounds where the concentration of aromatic compounds is much higher compared with the downstream-located Kohtla river from where PC18 was isolated (Heinaru *et al.*, 2000). Thus, according to the kinetic analysis of *p*-cresol degradation, strain PC24, which has a high substrate tolerance, expresses a high K_s and a low specific growth rate, while PC18, which has a low substrate tolerance and a low K_s, grows rapidly on *p*-cresol. Comparison of these results with our previous data on K_s values of strains PC18 and PC24 for phenol (Viggor *et al.*, 2008) revealed a positive correlation. We earlier verified that the diversity of phenol-oxidizing activity is due to differences in phenol hydroxylases, i.e. strains PC24 and PC18 harbor a single-component phenol hydroxylase and a multicomponent phenol hydroxylase, respectively (Merimaa *et al.*, 2006).

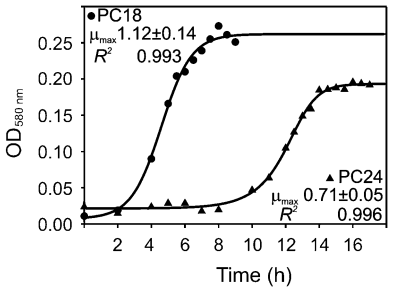

Fig. 2. Experimental and simulation data of PC18 and PC24 strain growth on 1.3 mM *p*-cresol. The Richards model was used to calculate the growth parameters (values are means ± SE). Experiments were repeated in triplicate, and results from a typical experiment from one culture are presented.

We suppose that the differences in whole-cell kinetic parameters between strains PC18 and PC24 revealed in this study are mainly caused by differences in the structure and regulation of expression of respective PCMH genes. This statement is also supported by the different growth yields of these strains on *p*-cresol (Fig. 2), despite the fact that both strains produced the same growth yields on *p*-hydroxyenzoate (Heinaru *et al.*, 2001). However, the different growth yields of these strains may have also been caused by different catechol cleavage enzymes. Namely, strain PC18 harbors only catechol 2,3-dioxygenase (*meta* pathway), but strain PC24 degrades aromatic compounds only via catechol 1, 2-dioxygenase (*ortho* pathway). Although both strains catabolized *p*-cresol via the protocatechuate *ortho* pathway, minor catechol 1,2-dioxygenase activity (Heinaru *et al.*, 2001) causes lactone accumulation and thus the low growth yield of strain PC24 can be explained. It is known that *ortho* fission reactions are rarely used by bacteria growing on methyl-substituted phenols due to the nonproductive accumulation of a nonmetabolizing methyl-substituted lactone.

Sequence analysis of the *pchRACXF* operon

In order to analyze the nucleotide sequences of the genes encoding PCMH and *p*-hydroxybenzaldehyde dehydrogenase in PC18 and PC24, a set of primers according to the corresponding sequences of *P. putida* NCIMB 9866 and *P. mendocina* KR1 were designed so that the resulting PCR products would partially overlap each of its neighbors.

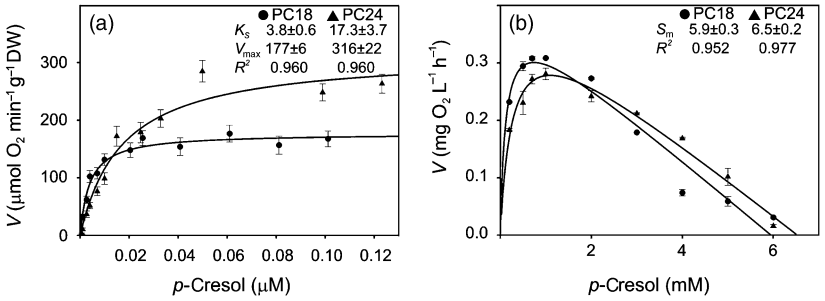

Fig. 3. Determination of apparent half-saturation constant, K_s, and maximum specific activity, V_{max}, (a) and maximum *p*-cresol inhibitory concentration, S_m (b) values from experimental data of *p*-cresol-dependent oxygen-consumption rate curves of strains PC18 and PC24 using Michaelis–Menten (a) and Luong (b) models. Error bars indicate the SD. Values of parameters are means ± SE.

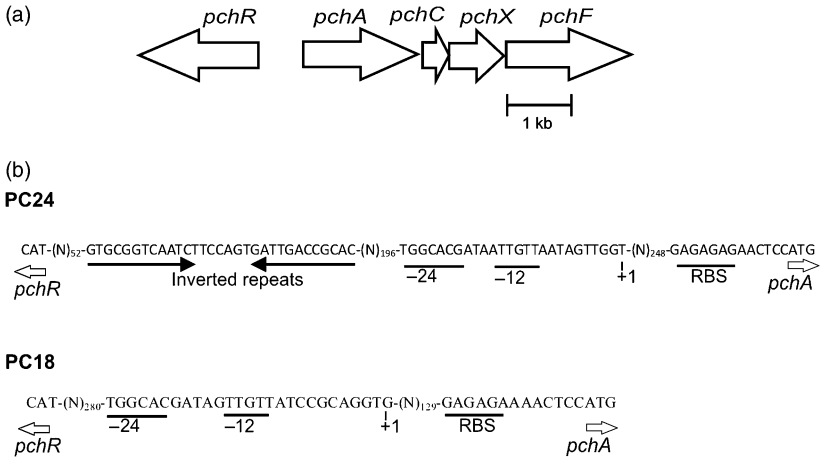

Fig. 4. (a) Genetic context of the *pchRACXF* gene cluster. (b) Schematic representation of the organization of the *pch* promoter region of strains PC18 and PC24. The arrows indicate inverted repeats, +1 indicates the start of transcription, and the putative −24 and −12 motifs and putative ribosome-binding sites are underlined.

The nucleotide sequences of the respective PCR products obtained using PC18 and PC24 as templates were aligned with the respective sequences of *P. putida* NCIMB 9866 and *P. mendocina* KR1, and further manually assembled. As a result, the nucleotide sequences of a 6573-bp region of strain PC24 and a 6457-bp region of strain PC18 were determined. For both sequences, five putative ORFs were identified (Fig. 4a) based on comparison of nucleotide and deduced amino acid sequences with the corresponding genes of *P. putida* NCIMB 9866 and *P. mendocina* KR1. According to BLAST searches, the latter two strains were revealed as closest matches for PC18 and PC24. In both strains, five putative ORFs were identified: *pchR* (1857 bp), putatively encoding a regulator of the *pchACXF* operon; *pchA* (1475 bp), encoding *p*-hydroxybenzaldehyde dehydrogenase; *pchC* (335 bp); *pchX* (692 bp), encoding a protein of unknown function; and *pchF* (1568 bp), encoding PCMH together with *pchC*. The ORFs were designated according to *P. putida* NCIMB 9866. The genes *pchA–pchF* putatively form one operon that is divergently transcribed from the *pchR* gene (Fig. 4a). The G+C content of the analyzed regions was about 60.2%.

Comparison of the deduced amino acid sequences of these five ORFs from PC18 and PC24 with the reference strains *P. putida* NCIMB 9866 and *P. mendocina* KR1 is presented in Table 2. These data show that the *pch* operons of strains PC18 and PC24 are more similar to *P. mendocina* KR1 than to *P. putida* NCIMB 9866. We also analyzed the structure of PchF of PC18 and PC24 and of reference strains KR1 and NCIMB 9866 using the programs MODBASE (http://modbase.compbio.ucsf.edu/ModWeb20-html/modweb.html) and PYMOL 0.99 (http://pymol.org/). The results showed that there are no substantial differences in the PchF structure between these strains, especially with regard to the substrate-binding cavity (data not shown). Thus, the different kinetic parameters of *p*-cresol degradation of PC18 and PC24 obtained in this study are probably not caused by differences in the structure of the PCMH protein.

Table 2. Comparison of the deduced amino acid sequences of different *pch* gene products (% amino acid identity is shown)

Comparison of strains	PchR	PchA	PchC	PchX	PchF
P. fluorescens PC18/*P. fluorescens* PC24	89	97	97	93	94
P. putida NCIMB 9866/*P. fluorescens* PC18	ND	83	56	49	79
P. putida NCIMB 9866/*P. fluorescens* PC24	ND	83	57	47	80
P. mendocina KR1/*P. fluorescens* PC18	78	90	77	63	83
P. mendocina KR1/*P. fluorescens* PC24	76	90	78	62	84
P. putida NCIMB 9866/*P. mendocina* KR1	ND	81	54	48	74

ND, not determined.

The structure of the promoter, containing the consensus sequence (TGGCAC-N_5-TTGCW) (Merrick, 1993) of σ^{54}-dependent promoters, was identified upstream of the *pchA* gene of both strains (Fig. 4b). However, these promoter

sequences differ from the consensus sequence, having T instead of C at position −12 (TGGCAC-N_5-TTGTT). In *P. mendocina* KR1, the nucleotide sequence TGGCAC-N_5-TTGTT lies upstream of the *pcuC* gene, in which there is also T at position −12. The −12 element, with the central consensus sequence TTGCW, contributes to binding affinity. The latter element may play a more complex role in RNA synthesis, beyond simply assisting in promoter recognition (Buck & Cannon, 1989; Tintut *et al.*, 1995; Wang *et al.*, 1997; Wang & Gralla, 1998). Prior studies have suggested that the −12 region sequences contribute to establishment of the basal transcription level (Wang *et al.*, 1997). The −12 region can contribute not only to transcription specificity but also to its regulatory response. Thus, a common feature of deregulated promoters is a loss of C at −12; thereby, this nucleotide would appear to be critical for fork junction binding (Wang *et al.*, 1999).

Operons of degradative bacteria that should work in bacteria-inhabiting polluted sites certainly need an efficient transcriptional control system (de Lorenzo & Pérez-Martín, 1996). All σ^{54}-dependent promoters analyzed so far are positively regulated by transcriptional activators that usually bind to specific DNA sequences located unusually far (between 100 and 200 bp) upstream of the promoter. The binding sequences are often inverted repeats that can be moved away by > 1 kb without losing their ability to mediate transcription (Kustu *et al.*, 1991; Morett & Segovia, 1993). Using a web-based program (http://www.proweb.org/proweb/Tools/selfblast.html), an inverted repeat sequence was found in the respective promoter–operator area of PC24, but not of PC18 (Fig. 4b). Also, the promoter–operator area of PC24 was 116 bp longer than of PC18 (Fig. 4b).

Mapping of the transcriptional start sites of the *pch* operons

To determine the transcriptional initiation sites of the *pch* operons of strains PC18 and PC24, putatively located in the *pchR–pchA* intergenic region, the 5′ RACE PCR technique was used on RNA extracted from cells grown on *p*-cresol. The nucleotide sequences of 5′ RACE PCR products indicated that transcription starts at thymine located 261 bases upstream of the *pchA* translational start site in PC24, and at guanine located 143 bases upstream of the translational start in PC18 (Fig. 4b). This provides further evidence that the above-discussed promoter sequences detected are indeed actual promoters of the *pch* operons in these strains.

Sequence analysis of PchR gene products

The amino acid sequences deduced from the *pchR* genes of PC18 and PC24 (nucleotide positions 1–1857 bp, 618 amino acids, putatively 67.4 kDa for PC24 and 67.8 kDa for PC18) were 89% identical (Table 2). Comparison of these sequences with translated nucleotide sequence entries in the GenBank database revealed the highest homology of PchR proteins with PcuR of *P. mendocina* KR1 (76–78% identity). TbuT of *Ralstonia pickettii* PKO1 (Byrne & Olsen, 1996), EugR of *Pseudomonas* sp. OPS1 (Brandt *et al.*, 2001), XylR of *P. putida* mt-2 (Inouye *et al.*, 1988), PhlR of *P. putida* H (Burchhardt *et al.*, 1997), MopR of *Acinetobacter calcoaceticus* NCIB8250 (Schirmer *et al.*, 1997), TouR of *Pseudomonas stutzeri* OX1 (Arenghi *et al.*, 1999), and DmpR of *Pseudomonas* sp. strain CF600 (Shingler *et al.*, 1993) showed 37–46% identity to PchR of PC18 and PC24. Among these sequences, TbuT and EugR are more similar to PchR. TbuT and EugR regulate operons involved in the catabolism of toluene and eugenol, respectively. Eugenol hydroxylase genes *ehyA* and *ehyB* have strong sequence similarities to the PCMH genes *pchC* and *pchF* from *P. putida* NCIMB 9866, respectively (Brandt *et al.*, 2001).

All of these proteins are members of the σ^{54}-dependent NtrC/XylR family of positive transcriptional activators (Morett & Segovia, 1993; Shingler, 1996). PchR proteins of PC18 and PC24 have all of the specific sequence characteristics of this family (Fig. 5). This type of regulator has a conserved four-part structure that includes an amino-terminal (A-domain) region linked to a central activation C-domain by a short B-domain, and a carboxyl-terminal DNA-binding D-domain (helix–turn–helix). The number of residues separating the C and D domains is highly variable within this family, and these regions bear little sequence identity (Shingler *et al.*, 1993).

An A-domain acts as the receiver module involved in the recognition of cognate environmental signals. This domain is poorly conserved and is most variable in length. Sequence identity in the effector-binding domain A of PchR proteins from PC18 and PC24 is 92% (19 amino acid differences) (Fig. 6). Figure 6 also shows the positions in XylR and DmpR where mutations affected inducer binding and that were closest to our amino acid differences. In XylR172, Glu (E) was mutated to Lys (K) (Delgado & Ramos, 1994), in XylR85, Pro (P) was mutated to Ser (S) (Delgado *et al.*, 1995), and in DmpR184, Arg (R) was mutated to Trp (W) (Shingler & Pavel, 1995).

The C-domain is the most conserved region among the proteins of this family, as it is involved in binding and hydrolysis of ATP that is basic for the activation of σ^{54} promoters (Delgado & Ramos, 1994; Shingler & Moore, 1994).

For phylogenetic analysis, a neighbor-joining tree based on deduced amino acid sequences of PchR of PC18 and PC24 was constructed alongside the σ^{54}-dependent regulator proteins accessible in GenBank (Fig. 7). Unfortunately, in *P. putida* NCIMB 9866, the regulator gene has not been sequenced. Our analysis indicated that PchR regulators of PC18 and PC24 and PcuR of KR1, which are all activated in response to the aromatic substrate *p*-cresol, constitute a separate cluster in

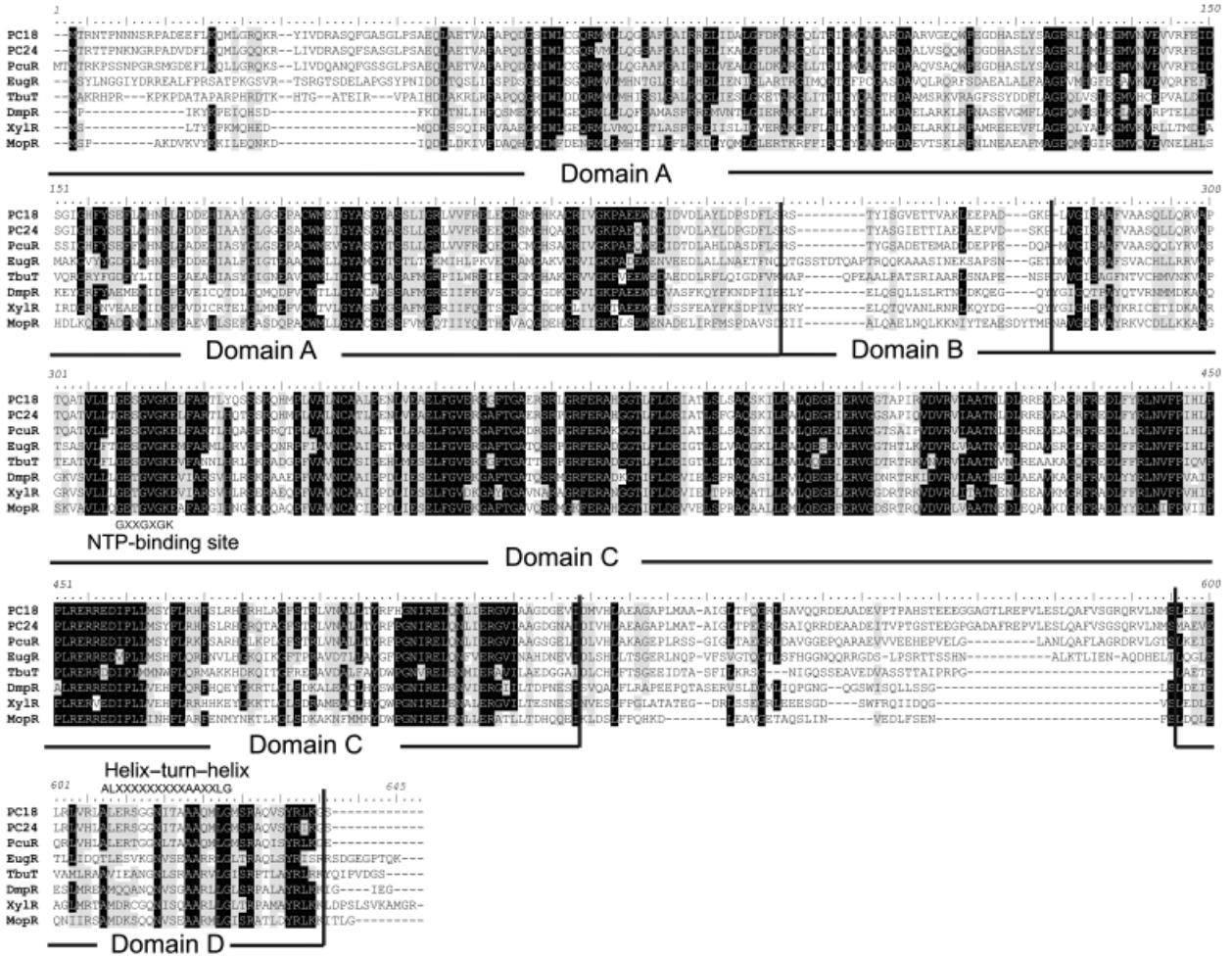

Fig. 5. Multiple sequence alignment of PchR from *Pseudomonas fluorescens* PC18 and PC24, PcuR from *Pseudomonas mendocina* KR1 (Ramos-González *et al*., 2002), EugR from *Pseudomonas* sp. OPS1 (Brandt *et al*., 2001), TbuT from *Ralstonia pickettii* PKO1 (Byrne & Olsen, 1996), XylR of *Pseudomonas putida* mt-2 (Inouye *et al*., 1988), PhlR of *P. putida* H (Burchhardt *et al*., 1997), MopR of *Acinetobacter calcoaceticus* NCIB8250 (Schirmer *et al*., 1997), TouR of *Pseudomonas stutzeri* OX1 (Arenghi *et al*., 1999), and DmpR of *Pseudomonas* sp. strain CF600 (Shingler *et al*., 1993). The amino acid residues conserved in all 10 sequences are indicated by white letters on a black background. The locations and boundaries of the four domains (described in the text) are outlined and labeled accordingly. The locations of the putative NTP-binding site and the helix–turn–helix motif along with their respective consensus sequences are also shown. Identities are shown by black boxes with white letters, similarities by gray boxes with black letters, and differences by white boxes with black letters.

the tree. Thus, they are far more similar to each other than to other well-known σ^{54}-dependent regulators.

Different induction patterns of PC18 and PC24 are caused by differences in the PchR regulators of these strains

To investigate whether the different induction patterns of the PchR regulators of PC18 and PC24 observed are caused by some specific structural features of these two proteins, a complementation assay in which *pchR* of PC18 was introduced into PC24 was constructed and the inducibility of the *pch* operon was analyzed in the resulting strain. For this assay, plasmid pP9TT$_B$-18RBS (18RBS) (which carries the entire *pchR* and the upstream promoter–operator area) was constructed as described in Materials and methods. This plasmid was electrotransformed into strain PC24, the resulting strain was grown overnight in liquid minimal medium supplemented with 2.5 mM phenol, and PCMH activity was determined. As shown in Fig. 8, *pch* operon expression was induced by phenol in PC24 bearing PchR of PC18. At the same time, in wild-type PC24 growing in phenol-containing medium as well as in CAA (uninduced conditions), PCMH activity was not found (Fig. 8). As a control, strain PC24 was complemented with construct ΔpP9TT$_B$-18RBS::km (PC24+18RBS/Km^r), in which most

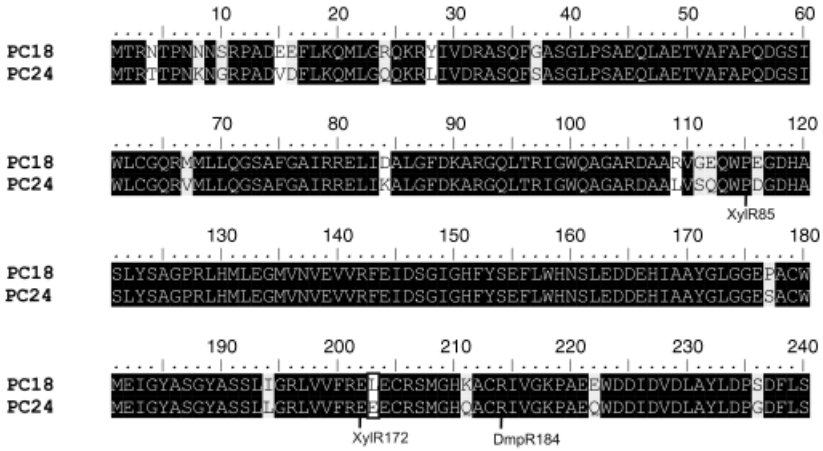

Fig. 6. Sequence alignment of the PchR A-domain from *Pseudomonas fluorescens* PC18 and PC24. The figure shows the positions in XylR and DmpR where mutations affected inducer binding and that were closest to our amino acid differences. In XylR172, Glu (E) was mutated to Lys (K) (Delgado & Ramos, 1994); in XylR85, Pro (P) to Ser (S) (Delgado *et al.*, 1995); and in DmpR184, Arg (R) to Trp (W) (Shingler & Pavel, 1995). Identities are shown by black boxes with white letters, similarities by gray boxes with black letters, and differences by white boxes with black letters.

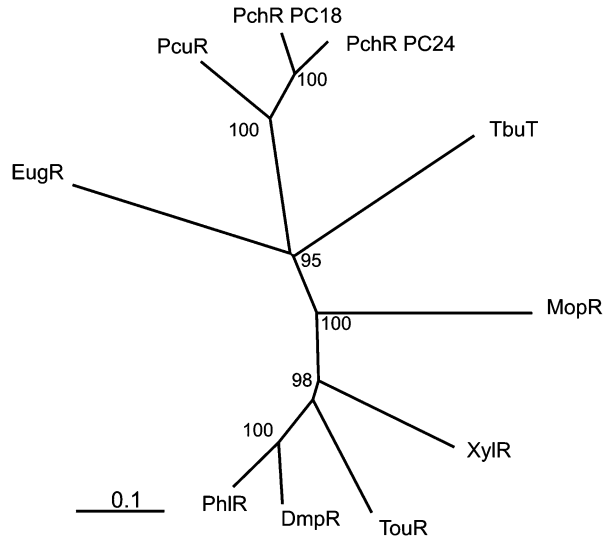

Fig. 7. A phylogenetic tree based on deduced amino acid sequences of σ^{54}-dependent regulators, PchR from *Pseudomonas fluorescens* PC18 and PC24, PcuR from *Pseudomonas mendocina* KR1 (Ramos-González *et al.*, 2002), EugR from *Pseudomonas* sp. OPS1 (Brandt *et al.*, 2001), TbuT from *Ralstonia pickettii* PKO1 (Byrne & Olsen, 1996), XylR of *Pseudomonas putida* mt-2 (Inouye *et al.*, 1988), PhlR of *P. putida* H (Burchhardt *et al.*, 1997), MopR of *Acinetobacter calcoaceticus* NCIB8u250 (Schirmer *et al.*, 1997), TouR of *Pseudomonas stutzeri* OX1 (Arenghi *et al.*, 1999), and DmpR of *Pseudomonas* sp. strain CF600 (Shingler *et al.*, 1993). Bootstrap values (per 100 trials) higher than 50% are indicated at the nodes. The scale bar represents 0.1 substitutions per amino acid site.

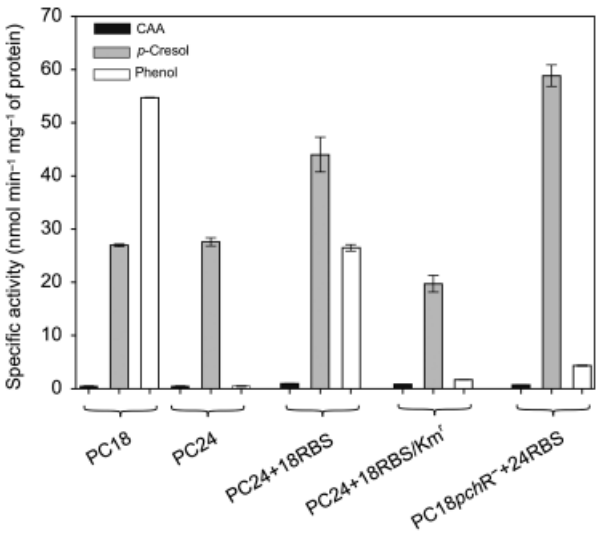

Fig. 8. Expression of PCMH activities (nmol min^{-1} mg^{-1} of protein) in cell-free extracts of strains PC18 and PC24; and constructs PC24+18RBS, PC24+18RBS/Km^r, and PC18*pchR*$^-$+24RBS during growth on *p*-cresol, phenol, and CAA. Mean values for independent cultures are shown with SD.

of the *pchR* was deleted and the induction of PCMH activity with phenol was not found.

In addition, a complementation assay was conducted in which the native *pchR* regulator in strain PC18 was inactivated by Km^r gene insertion (constructed as described in Materials and methods). As expected, after complementation of PC18*pchR*$^-$ with pP9TT$_B$-24RBS (24RBS), PCMH activity was induced only with *p*-cresol (Fig. 8). Based on these results, we conclude that differences in the amino acid sequences of PchR regulators of the two studied strains led to different effector-binding capabilities of these proteins. Phenol is a more efficient effector molecule for PchR of PC18 than *p*-cresol, but it does not activate the regulator of PC24. At the same time, both regulators respond similarly to *p*-cresol.

The capability of bacteria to adapt to certain contaminated environments by enhancing degradative capacities has been shown to be caused by mutational change within the effector-binding subregion of DmpR (Sarand *et al.*, 2001). Also, Delgado & Ramos (1994) have shown that due to a single

amino acid change at the N-terminal end of XylR, the protein acquired the ability to bind a new effector not recognized by the wild-type protein. This and other studies (Pavel *et al.*, 1994; Delgado *et al.*, 1995; Fernández *et al.*, 1995; Shingler & Pavel, 1995; Pérez-Martín & de Lorenzo, 1996; Salto *et al.*, 1998; Skärfstad *et al.*, 2000; Wise & Kuske, 2000; O'Neill *et al.*, 2001; Sarand *et al.*, 2001; Solera *et al.*, 2004; Galvão & de Lorenzo, 2006) support the regulatory noise hypothesis to describe how transcriptional regulators may evolve competence to deal with novel environmental signals (de Lorenzo & Pérez-Martín, 1996; Garmendia *et al.*, 2001).

Our results demonstrate that strong selective pressure and competition under natural mixed substrate conditions might lead to the evolution of different regulatory mechanisms for the expression of highly similar degradation genes in phylogenetically related bacterial strains. In the cases of PC18 and PC24, changes in *pchR* regulatory genes have led to different expression patterns of catabolic routes to overcome potential metabolic conflicts during degradation of phenol and *p*-cresol mixtures.

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