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 $\sigma^{54}$-dependent regulator, was shown. Although the promoters of these operons resembled $\sigma^{54}$-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 (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 $\beta$-ketoapidate 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 $\alpha$ subunits contain an active site FAD covalently linked to a tyrosine residue, whereas the $\beta$ 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; Rudolph 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.
The best-characterized PCMH is the plasmid-encoded PCMH69B genes of P. putida NCIMB 9865 and pcuCAXB in P. mendocina KR1), with pchA/pcuA 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 pchA operon, encodes the transcriptional regulator of this operon (Ramos-González et al., 2002).

The best-characterized PCMH is the plasmid-encoded PCMH69 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 PCMH69B 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 (Bauchoe & Eldsen, 1960) supplemented with either phenol (2.5 mM) or p-cresol (1.3 mM). Escherichia coli DH5α harboring pTZ57R/T- or pPR9TTB-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 (μmax) 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 described by Viggior et al. (2008). The apparent kinetic constants, half saturation constant (Kₘ), and maximum specific activity (Vₘₐₓ), were determined using a nonlinear regression method according to the Michaelis–Menten equation (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...
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 CO₂ 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:

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

where \( V_{\text{max}} \) 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 3730xl 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 1.8.3 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.

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**Table 1.** Bacterial strains and plasmids used for this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or construction</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC18 biotype B</td>
<td>Wild-type strain, Phex⁺, pcR⁺</td>
<td>Heinaru et al. (2001)</td>
</tr>
<tr>
<td>PC24 biotype C</td>
<td>Wild-type strain, Phex⁺, pcR⁺</td>
<td>Merimaa et al. (2006)</td>
</tr>
<tr>
<td>PC18pchR⁺</td>
<td>Native <em>pcR</em> of strain PC18 knockout derivative with Km⁺</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5感受器</td>
<td>supE44 ΔlacU169 (op80 lacZ ΔM15) recA1 endA1 hsdR17 thi-1 gyrA96 relA1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>HB101</td>
<td>subE44 subF58 hsdR3 (r6 m2 Δ你会发现) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</td>
<td>Boyer &amp; Roulland-Dussoix (1969)</td>
</tr>
<tr>
<td>CC118Δpir</td>
<td>Δ ara-leaf) araD ΔlacX74 galE galK phoA20 thi-1 rpsE ropA argE (Amr) recA1, λpir phage lysogen</td>
<td>Herrero et al. (1990)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTZ57R/T</td>
<td>Cloning vector (Ap⁺)</td>
<td>MBI Fermentas</td>
</tr>
<tr>
<td>pUTmini-Tn5 Km2</td>
<td>Delivery plasmid for homologous recombination (Ap⁺)</td>
<td>De Lorenzo et al. (1990)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Helper plasmid for conjugal transfer of pGP704 L (Km⁺)</td>
<td>Figurski &amp; Helinski (1979)</td>
</tr>
<tr>
<td>pPR9TTB</td>
<td>Low-copy-number lacZ-based promoter probe plasmid pPR9TT, derivative without the lacZ gene; Cm⁺, Ap⁺</td>
<td>Kivistik et al. (2006)</td>
</tr>
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<td>PC18pTZ57R</td>
<td>pTZ57R/T containing the PCR-amplified <em>pcR</em> gene of strain PC18</td>
<td>This study</td>
</tr>
<tr>
<td>pTZ57RΔ18pchR::km</td>
<td><em>pcR</em> of strain PC18 in pTZ57R/T is interrupted with Km⁺ gene from pUTmini-Tn5 Km2 by replacing Bsp1407I- and BglII-generated fragment from <em>pcR</em> by Km⁺ gene</td>
<td>This study</td>
</tr>
<tr>
<td>pGP704AΔ18pchR::km</td>
<td>pGP704 L with SpI-SacI fragment of 18pchR::km from pTZ57R/A18pchR::km in the vector plasmid opened with the same reductases</td>
<td>This study</td>
</tr>
<tr>
<td>pTZ57R/T-18RBS</td>
<td>pTZ57R/T containing the PCR-amplified <em>pcR</em> gene and the upstream promoter–operator area of strain PC18</td>
<td>This study</td>
</tr>
<tr>
<td>pTZ57R/T-24RBS</td>
<td>pTZ57R/T containing the PCR-amplified <em>pcR</em> gene and the upstream promoter–operator area of strain PC24</td>
<td>This study</td>
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<td>pPR9TTB-18RBS</td>
<td>pPR9TTB with HindIII-Xbal fragment of 18RBS from pTZ57R/T-18RBS in the vector plasmid opened with the same reductases</td>
<td>This study</td>
</tr>
<tr>
<td>pPR9TTB-18RBS</td>
<td>pPR9TTB with HindIII-Xbal fragment of 24RBS from pTZ57R/T-24RBS in the vector plasmid opened with the same reductases</td>
<td>This study</td>
</tr>
<tr>
<td>ΔpPR9TTB-18RBS:km</td>
<td>18RBS in pPR9TTB is interrupted with the Km⁺ gene from pUTmini-Tn5 Km2 by replacing Bsp1407I- and Xbal-generated fragment from <em>pcR</em> by the Km⁺ gene</td>
<td>This study</td>
</tr>
</tbody>
</table>

Km⁺, resistance to kanamycin; Phex⁺, pcR⁺, the ability to degrade phenol and p-cresol, 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 exponential-phase cultures using the Nucleospin® RNA II extraction kit (Macherey-Nagel), according to the manufacturer’s instructions. DNATase 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′-GGCAGGGTCAGCAAGG-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(dC) tailing with terminal deoxynucleotidyl 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′-GGGACCGGTCGACTATGACCTG13-D 3′), the pch-specific primer RA2 (5′-CAGTTCAAGGTTTGGATAGGCTG-3′), and primer Arb2 (5′-GGCCACGGGTCCAG TAGTAGCC-3′) (Caetano-Anollés, 1993). The primers Arb1 (5′-GGGACCGGTCGAC TAGTACNNNN-3′) and Arb2 (Caetano-Anollés, 1993) were also used to analyze the ends of the genes pchR and pchE. 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/Adom™ 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′-GCGTGATCTGCAGCAAGC-3′) and pchR5 (5′-GTTTACACTGGCAGA-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′ gene. A fragment of approximately 1 kb of the pchR gene was deleted. The Km′ gene was amplified by PCR from the plasmid pUTmini-Tn5 Km2 using the primer KmSac (5′-CAGGAGCTCGTGGTAACTTTAACAAGACCC-3′) (Hörak et al., 2004). The Ecl136II-cleaved DNA fragment containing the Km′ gene was inserted into the cleaved pchR gene of strain PC18. The Bsp1407I and BglII ends were blunt ended before ligation. The resulting plasmid pGP704A18pchR::km sequence from pTZ57R/A18pchR::km excised and inserted as the SphI–Sacl fragment plasmid pGP704. The resulting plasmid pGP704A18pchR::km was conjugatively transferred from E. coli CC118pir (Herrero et al., 1990) into P. fluorescens strain PC18 using the helper plasmid pRK2013 (Figurski & Helinski, 1979). Strain PC18pchR was verified by PCR analysis using the primers KmOcc (5′-TCGAGCAAGCACTTTCCC-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′-CTCTTTATTGCGGAAAGGCGATC-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 pPR9TTB. After verification of the constructs by digestion with HindIII and XbaI, pPR9TTB-18RBS was electrotransformed into strain PC24 and the transformants were selected on LB medium with carbenicillin (5000 μg mL −1).

pPR9TTB-24RBS was electrotransformed into PC18pchR strain. 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′-AATTGCTTTGAACACTTTGACGAGA-3′) and pchRBS.

Mutant ΔpchR of PC18::km was constructed by restriction of pchR with Bsp1407 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 KmOcc and pchRBS. Construct ΔpchR of PC18::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 K₂HPO₄–KH₂PO₄ 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 (ε = 21 cm² μmol⁻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 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 M) 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 Kohlta 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).

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-hydroxybenzoate (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 habors only catechol 2,3-dioxygenase (meta pathway), but strain PC24 degrades aromatic compounds only via catechol 1, 2-dioxygenase (ortho pathway). Although both strains catalyzed 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.
The nucleotide sequences of the respective PCR products obtained using PC18 and PC24 as templates were aligned with the respective sequences of \textit{P. putida} NCIMB 9866 and \textit{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 \textit{P. putida} NCIMB 9866 and \textit{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: \textit{pchR} (1857 bp), putatively encoding a regulator of the \textit{pchACXF} operon; \textit{pchA} (1475 bp), encoding \(p\)-hydroxybenzaldehyde dehydrogenase; \textit{pchC} (335 bp); \textit{pchX} (692 bp), encoding a protein of unknown function; and \textit{pchF} (1568 bp), encoding PCMH together with \textit{pchC}.

The ORFs were designated according to \textit{P. putida} NCIMB 9866. The genes \textit{pchA}–\textit{pchF} putatively form one operon that is divergently transcribed from the \textit{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 \textit{P. putida} NCIMB 9866 and \textit{P. mendocina} KR1 is presented in Table 2. These data show that the \textit{pch} operons of strains PC18 and PC24 are more similar to \textit{P. mendocina} KR1 than to \textit{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.

The structure of the promoter, containing the consensus sequence (TGGCAC-N\textsubscript{5}-TTGCW) (Merrick, 1993) of \(s\textsubscript{54}\)-dependent promoters, was identified upstream of the \textit{pchA} gene of both strains (Fig. 4b). However, these promoter

\noindent Table 2. Comparison of the deduced amino acid sequences of different \textit{pch} gene products (% amino acid identity is shown)

<table>
<thead>
<tr>
<th>Comparison of strains</th>
<th>\textit{PchR}</th>
<th>\textit{PchA}</th>
<th>\textit{PchC}</th>
<th>\textit{PchX}</th>
<th>\textit{PchF}</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{P. fluorescens} PC18/\textit{P. fluorescens} PC24</td>
<td>89</td>
<td>97</td>
<td>97</td>
<td>93</td>
<td>94</td>
</tr>
<tr>
<td>\textit{P. putida} NCIMB 9866/\textit{P. fluorescens} PC18</td>
<td>ND</td>
<td>83</td>
<td>56</td>
<td>49</td>
<td>79</td>
</tr>
<tr>
<td>\textit{P. putida} NCIMB 9866/\textit{P. fluorescens} PC24</td>
<td>ND</td>
<td>83</td>
<td>57</td>
<td>47</td>
<td>80</td>
</tr>
<tr>
<td>\textit{P. mendocina} KR1/\textit{P. fluorescens} PC18</td>
<td>78</td>
<td>90</td>
<td>77</td>
<td>63</td>
<td>83</td>
</tr>
<tr>
<td>\textit{P. mendocina} KR1/\textit{P. fluorescens} PC24</td>
<td>76</td>
<td>90</td>
<td>78</td>
<td>62</td>
<td>84</td>
</tr>
<tr>
<td>\textit{P. putida} NCIMB 9866/\textit{P. mendocina} KR1</td>
<td>ND</td>
<td>81</td>
<td>54</td>
<td>48</td>
<td>74</td>
</tr>
</tbody>
</table>

ND, not determined.
sequences differ from the consensus sequence, having T instead of C at position –12 (TGGCAC-N3-TTGTT). In P. mendocina KR1, the nucleotide sequence TGGCAC-N3-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 transcription specificity but also to its regulatory response. Thus, a common feature of deregulated promoters is a loss of C at –12 (TGGCAC-N3-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 transcription specificity but also to its regulatory response.

Positive 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 (Küst 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). TuB ofRalstonia pickettii PK01 (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, TuB and EugR are more similar to PchR. TuB 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 σ54-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 induced 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 Thr (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 σ54 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 σ54-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
Thus, they are far more similar to each other than to other well-known σ54-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 pP9TTB-18RBS (18RBS) (which carries the entire pchR and the upstream promoter–operator area) was constructed as described in Materials and methods. This plasmid was electrotansformed 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 ΔpP9TTB-18RBS::km (PC24 + 18RBS/Km4), in which most
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 Kmr gene insertion (constructed as described in Materials and methods). As expected, after complementation of PC18pchR with pP9TTB-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; Skärstad 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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