

# Regulation of the *p*-hydroxybenzoic acid hydroxylase gene (*pobA*) in plant-growth-promoting *Pseudomonas putida* WCS358

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**The regulation of the *p*-hydroxybenzoate hydroxylase gene (*pobA*) of *Pseudomonas putida* WCS358 involved in the catabolism of *p*-hydroxybenzoic acid (PHB) to the central intermediate protocatechuate was studied. Protocatechuic acid (PCA) is then degraded via the  $\beta$ -ketoadipate pathway to form tricarboxylic acid intermediates. In several Gram-negative bacteria *pobA* has been found genetically linked to a regulator called *pobR* which activates *pobA* expression in response to PHB. In this study the identification and characterization of the *pobC-pobA* locus of *P. putida* WCS358 is presented. The *p*-hydroxybenzoate hydroxylase (PobA) is highly identical to other identified PobA proteins, whereas the regulatory protein PobC did not display very high identity to other PobR proteins studied and belonged to the AraC family of regulatory proteins, hence it has been designated PobC. Using the *pobA* promoter transcriptionally fused to a promoterless *lacZ* gene it was observed that induction via PobC occurred very efficiently when PHB was present and to a lesser but still significant level also in the presence of PCA. This PobC-PCA response was genetically demonstrated by making use of *pobC::Tn5* and *pcaH::Tn5* mutants of strain WCS358 constructed in this study. In *pobC* mutants both the *p*-hydroxybenzoic and PCA response were not observed, whereas in the *pcaH* mutant, which lacks a functional protocatechuate 3,4-dioxygenase, the protocatechuic-acid-dependent *pobA* activation was still observed. Finally, the activation of *pobA* by PHB varied according to the concentration and it was observed that in the *pcaR::Tn5* regulatory mutant of strain WCS358 the *pobA* promoter activity was reduced. PcaR is a regulator involved in the regulation of several loci of the  $\beta$ -ketoadipate pathway, one of which is *pcaK*. It was postulated that the reduction of *pobA* activation in *pcaR::Tn5* mutants was because there was no expression of the *pcaK* gene encoding the PHB transport protein resulting in lower levels of PHB present inside the cell.**

Keywords: protocatechuic acid, *pobA*, *pobC*, aromatic acid

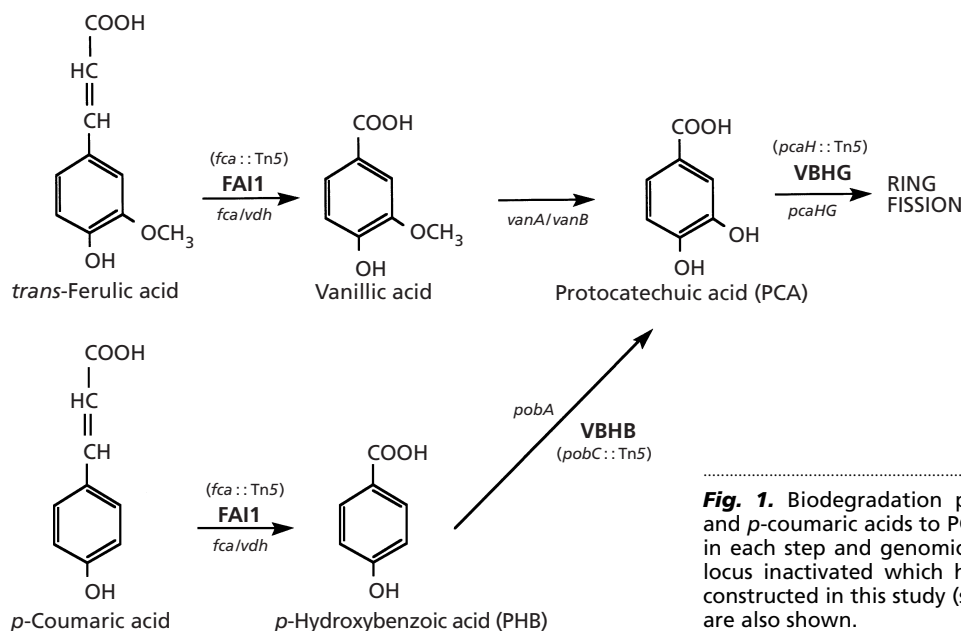
## INTRODUCTION

Pseudomonads have the capacity to catabolize a large number of low-molecular-mass aromatic compounds. Many of these compounds like ferulic acid, vanillic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid (PHB) and

protocatechuic acid (PCA) arise in the environment from the biodegradation of lignin. These compounds are also present in plant cells and consequently are abundantly present in the rhizosphere and have been found to play an important role in signalling gene expression between plants and *Agrobacterium tumefaciens* (Bolton *et al.*, 1986; Melchers *et al.*, 1989), demonstrating that these molecules can act as signals for bacteria when in close proximity to the plant. Many strains belonging to the three species of fluorescent pseudomonads, *Pseudomonas putida*, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*, are aggressive

**Abbreviations:** PCA, protocatechuic acid; PHB, *p*-hydroxybenzoic acid.

The GenBank/EMBL/DDBJ accession numbers for the *pcaR*, *pobC-pobA* and *pcaHG* sequences reported in this paper are AJ252090, AJ251792 and AJ295623, respectively.



**Fig. 1.** Biodegradation pathway of ferulic and *p*-coumaric acids to PCA. Genes involved in each step and genomic mutants having a locus inactivated which have been used or constructed in this study (see text for details) are also shown.

rhizosphere colonizers and plant-growth-promoting (O'Sullivan & O'Gara, 1992), thus they have evolved chemotactic and sensing systems to reach and colonize the rhizosphere. Plant phenolic compounds could play a role in the establishment of the symbiosis of plants with plant-growth-promoting bacterial strains, thus the study of catabolic and regulatory genes responding to these compounds could be an approach to investigate this possible role.

In bacteria, diverse aromatic compounds are initially transformed to a limited number of central intermediates, namely catechol (or substituted derivatives) and PCA. These compounds are then channelled into two possible ring fission pathways, either the *ortho*- or *meta*-cleavage pathway, funnelling these compounds into the tricarboxylic acid cycle (van der Meer *et al.*, 1992). In Gram-negative bacteria, ferulic acid is initially degraded to PCA via vanillic acid, whereas coumaric acid is degraded via PHB (Toms & Wood, 1970; Venturi *et al.*, 1998) (Fig. 1). These catabolic conversion steps require at least three genetic loci (Fig. 1). The transformation of ferulic acid to vanillic acid involves an operon encoding an enoyl-CoA hydratase/aldolase, a vanillin dehydrogenase and another gene encoding feruloyl coenzyme A synthetase (Overhage *et al.*, 1999b; Priefert *et al.*, 1997; Venturi *et al.*, 1998). Vanillic acid is then degraded to PCA by a demethylase encoded by an operon consisting of two genes designated *vanA* and *vanB* (Brunel & Davison, 1988; Priefert *et al.*, 1997; Venturi *et al.*, 1998). The degradation of *p*-coumaric acid to PHB also requires at least one locus that transforms ferulic acid to vanillic acid (Venturi *et al.*, 1998), whereas the conversion of PHB to PCA requires a hydroxylase encoded by the *pobA* gene (Wong *et al.*, 1994; Parke, 1996; this study). All three loci involved in these conversion steps have been cloned and charac-

terized in plant-growth-promoting *P. putida* WCS358 (Venturi *et al.*, 1998; this study).

Several regulatory genes and proteins sensing and responding to such aromatic compounds have been identified and characterized (Kok *et al.*, 1998; Parke, 1993; Romero-Steiner *et al.*, 1994). In *Acinetobacter* sp. ADP1 two transcriptional activators have been identified: PobR, which elicits transcription of the *pobA* gene in response to PHB, and PcaU, which is triggered by PCA and switches on the expression of the set of genes required for the catabolism of protocatechuate (Gerischer *et al.*, 1998). The two regulators PobR and PcaU are 54% identical belonging to the same family (IcLR) and respond to closely related aromatic compounds (Kok *et al.*, 1998). Similarly, a transcriptional activator called PcaR has been described in *P. putida* and senses and responds to  $\beta$ -keto adipate, an intermediate in the degradation of PCA, and in turn switches on the transcription of three loci involved in the catabolism of protocatechuate (Romero-Steiner *et al.*, 1994).

The regulation of *pobA* expression by PobR has been extensively studied in *Acinetobacter* sp. ADP1 at the genetic and molecular level, and the PHB response and the DNA target have been investigated (DiMarco & Ornston, 1994; Kok *et al.*, 1997). In this study we present genetic data on the regulation of *pobA* expression in plant-growth-promoting *P. putida* WCS358. The *pobA-pobC* locus has been identified. The genetically linked regulator exhibited low similarity to the other studied PobR regulators and belonged to the AraC family of regulators, hence it was designated PobC. PobC activated *pobA* expression in response to PHB. Interestingly, it also had weak activity in the presence of PCA. Finally, it was observed that the regulator PcaR was important for efficient activation of *pobA* expression and it was

postulated that this effect was mediated via the expression of *pcaK*, the gene encoding a PHB transporter protein.

## METHODS

**Bacterial strains, plasmids and media.** Strains used in this study include *Escherichia coli* DH5 $\alpha$  (Hanahan, 1983), XL-1 Blue (Bullock *et al.*, 1987), JM101 (Messing, 1983) and HB101::Tn5 (Moore *et al.*, 1984). *P. putida* strains used are listed in Table 1, *P. aeruginosa* PAO1 was also used (B. Holloway collection). Plasmids used are listed in Table 1. *E. coli* was grown in LB medium (Miller, 1972) at 37 °C, whereas *Pseudomonas* strains were grown in LB medium or M9 minimal medium at 30 °C (Maniatis *et al.*, 1982). The following antibiotic concentrations were used ( $\mu\text{g ml}^{-1}$ ): tetracycline, 10 (*E. coli*) and 40 (*Pseudomonas*); kanamycin, 100; nalidixic acid, 25; ampicillin, 100; gentamicin, 10 (*E. coli*) and 40 (*Pseudomonas*). The promoter fusion hybrid plasmid pPPOBA was constructed as follows. The promoter of the *pobA* gene was PCR-amplified from pCOSHb as template

using two oligonucleotides, 5'-GGAATTCCGGGGATGCC-TGTCCGTTAAT-3' and 5'-GCTCTAGAGCTGTTGTTG-TCTCTAGTCGGG-3', and cloned into pUC18 to yield pPIBV1. After verification via sequence analysis it was then cloned as a 183 bp *EcoRI*-*XbaI* fragment (restriction sites indicated in bold type) into pMP220. The promoter of *pcaK* was cloned in promoter probe vector pMP220 as follows. Using two oligonucleotides (5'-CTCTAGAGTTGTTGTT-ATCCAGAATTAG-3' and 5'-GGAATTCCTTATCG-AACACGCCCTG-3') a 298 bp fragment consisting of DNA upstream the ATG start codon of *pcaK* was PCR-amplified using pCOS272 as template and cloned into pUC18, yielding pPKP1. This fragment was then removed from pPKP1 as an *EcoRI*-*XbaI* fragment and cloned into the corresponding sites of pMP220, yielding pPPCAK. The *pcaH* gene of *P. aeruginosa* PAO1 was located in contig 1 at position 174773–175492 (www.pseudomonas.com) and was amplified using two oligonucleotides (5'-GGAATTCATGACAGACGCCGAAAGC-3' and 5'-CGGGATCCGTCAGGCGTTCACGAAGAA-3') and PAO1 genomic DNA as template and cloned into pUC18, yielding pPAOH1. The *pcaR* gene was found in the PAO1 genome (www.pseudomonas.com) located in contig 1 at

**Table 1.** Plasmids and *P. putida* strains used

Strain or plasmid	Relevant characteristic*	Reference
<b>Strains</b>		
<i>P. putida</i> WCS358	Wild-type, rhizosphere isolate	Geels & Schippers (1983)
<i>P. putida</i> VBHB	Strain WCS358 <i>pobC</i> ::Tn5	This study
<i>P. putida</i> VBPC	Strain WCS358 <i>pcaR</i> ::Tn5	This study
<i>P. putida</i> VBHG	Strain WCS358 <i>pcaH</i> ::Tn5	This study
<i>P. putida</i> FAI1	Strain WCS358 <i>fca</i> ::Tn5	Venturi <i>et al.</i> (1998)
<b>Plasmids</b>		
pUC18	ColE1 replicon, Ap <sup>r</sup>	Yanisch-Perron <i>et al.</i> (1985)
pBlueScript KS	ColE1 replicon, Ap <sup>r</sup>	Stratagene
pBlueScript SK	ColE1 replicon, Ap <sup>r</sup>	Stratagene
pRK2013	ColE1 replicon, Km <sup>r</sup> Tra <sup>+</sup> Mob <sup>+</sup>	Figursky & Helinsky (1979)
pPH1JI	IncP1, Gm <sup>r</sup>	Beringer <i>et al.</i> (1978)
pMP220	Promoter probe vector IncP1, Tc <sup>r</sup>	Spaink <i>et al.</i> (1987)
pLAFR3	Broad-host-range cloning vector IncP1, Tc <sup>r</sup>	Staskawicz <i>et al.</i> (1987)
pPAOPCAR	pUC18 containing <i>pcaR</i> of <i>P. aeruginosa</i>	This study
pCOS272	pLAFR3 containing WCS358 DNA	This study
pBKP2.2	pBlueScript KS containing 2.2 kb <i>KpnI</i> fragment from pCOS272	This study
p2726.5	pBlueScript KS containing 6.5 kb <i>HindIII</i> fragment of WCS358 DNA from pCOS272	This study
p2726.5::Tn5	p2726.5 with Tn5 insertion in <i>pcaR</i>	This study
pCOS272::Tn5	pCOS272 with Tn5 insertion in <i>pcaR</i>	This study
pCOSHb	pLAFR3 containing WCS358 DNA	This study
pCOSHG1	pLAFR3 containing WCS358 DNA	This study
pCOSHG5	pLAFR containing the 5 kb <i>HindIII</i> fragment of pCOSHG	This study
pCOSHG1::Tn5	pCOSHG5 containing a Tn5 insertion in <i>pcaH</i>	This study
pBGH18	<i>P. putida</i> WCS358 <i>pcaGH</i> locus in pBlueScript	This study
pB4.7KN	pBlueScript KS containing a 4.7 kb <i>KpnI</i> - <i>NotI</i> fragment from pCOSHb	This study
pPAOH1	<i>pcaH</i> gene of <i>P. aeruginosa</i> in pUC18	This study
pPIBV1	<i>pobA</i> promoter in pUC18	This study
pPKP1	<i>pcaK</i> promoter in pUC18	This study
pPPCAK	<i>pcaK</i> promoter in pMP220	This study
pPPOBA	<i>pobA</i> gene promoter in pMP220	This study

\* Ap<sup>r</sup>, Km<sup>r</sup>, Sm<sup>r</sup>, Tc<sup>r</sup>, Gm<sup>r</sup>: resistance to ampicillin, kanamycin, streptomycin, tetracycline and gentamicin, respectively.

position 176294–177154 and was PCR-amplified from PAO1 genomic DNA using the primers 5'-GTGACCCGAGCCCGA-3' and 5'-TCAGGTGAACAGGCGCG-3' and cloned as a 860 bp DNA fragment into pUC18 to yield pPAOPCAR.

**Recombinant DNA techniques.** Digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 DNA ligase, end-filling with the Klenow fragment of DNA polymerase, hybridizations, radioactive labelling by random priming and transformation of *E. coli* were performed as described by Maniatis *et al.* (1982). Southern hybridizations were performed using Hybond-N+ membrane (Amersham Pharmacia Biotech). Plasmids were purified as described by Birnboim (1983) and with Qiagen columns. Total DNA from *Pseudomonas* strains was isolated by Sarkosyl/Pronase lysis as described by Better *et al.* (1983). Triparental matings between *E. coli* and *Pseudomonas* strains were performed with the helper strain *E. coli*(pRK2013) (Figurski & Helinski, 1979). The DNA sequence flanking the transposon insertion in mutant *P. putida* VBHB was determined using arbitrary PCR (Caetano-Annoles, 1993). In this procedure the DNA flanking the Tn5 insertion site is amplified in two rounds using primers specific to the ends of the Tn5 element and primers of random sequence which anneal to chromosomal sequences flanking the transposon. The technique and primers used were as described by O'Toole & Kolter (1998).

**Reporter gene fusion assays and analysis of substrate metabolism.**  $\beta$ -Galactosidase activity was determined essentially as described by Miller (1972) with the modifications of Stachel *et al.* (1985). The biodegradation of aromatic acids by *P. putida* WCS358 and Tn5 mutants was analysed by reverse-phase HPLC (RP-HPLC), using a Varian 9010 solvent delivery system equipped with a Varian 9050 UV/vis detector. *Pseudomonas* was grown either in LB medium supplemented with 0.15% (w/v) of the aromatic acid, or in M9 minimal medium supplemented with 0.1% and/or 0.05% of the aromatic acid. Samples were withdrawn from cultures after growth and centrifuged at 12000 g. The supernatant was diluted 100-fold into methanol and filtered through 0.2  $\mu$ m filters; 10  $\mu$ l samples were loaded on a 5  $\mu$ m spherical C18 reverse-phase column (Supelcosil LC18 150  $\times$  4.6 mm; Supelco) and eluted with 35% methanol and 65% water with 0.1% acetic acid at a flow rate of 0.8 ml min<sup>-1</sup>. The eluted metabolites were detected at 279 nm. The catabolic intermediates were identified by comparing their elution times with those of pure standards.

**Isolation of mutants unable to use PHB as carbon source and their complementation with a *P. putida* WCS358 gene bank.** Mutants unable to use PHB as carbon source but retaining the ability to use PCA, were screened as follows. Colonies from a Tn5 genomic mutant bank (Marugg *et al.*, 1985) were grown at 30 °C in duplicate on minimal M9 plates containing kanamycin and either PHB or PCA as sole carbon source. Mutants were selected which were unable to grow on PHB but retained the ability to grow on PCA. Complementation was performed via triparental matings between a cosmid gene library of *P. putida* WCS358, an *E. coli*(pRK2013) mobilizer (about 4  $\times$  10<sup>9</sup> cells each) and the WCS358 Tn5 mutant (2  $\times$  10<sup>8</sup> cells) were set up on a 0.45  $\mu$ m membrane filter (Millipore) on an LB plate. After 12 h incubation at 30 °C, cells were resuspended and plated on minimal M9 plates containing PHB (0.15%) with tetracycline.

**Cloning of the *pcaHG* and *pcaR* loci of *P. putida* WCS358.** The *pcaHG* genes of strain WCS358 were cloned using the *pcaH* gene from *P. aeruginosa* PAO1 as probe against the cosmid

gene library. A 736 bp *EcoRI*–*Bam*HI fragment from pPAOH1 was used as probe and a cosmid designated pCOSHG1 was identified that harboured the *pcaHG* locus on a 5 kb *Hind*III fragment. The genes were then further localized on a 1.8 kb *EcoRI*–*Hind*III subfragment. This fragment was cloned in pBluescript to yield pBGH18. The 5 kb *Hind*III fragment containing the *pcaHG* locus was cloned in pLAFR3 to yield pCOSHG5.

The *pcaR* gene of *P. putida* WCS358 was also cloned using the *pcaR* gene of *P. aeruginosa* PAO1 as probe to screen a cosmid bank of *P. putida* WCS358. An 875 bp *EcoRI*–*Hind*III fragment of pPAOPCAR was used as probe and a cosmid, designated pCOS272, was identified which harboured the *pcaR* gene on a 6.5 kb *Hind*III fragment. This fragment was cloned in pBluescript KS to yield p2726.5. The *pcaR* gene was further localized in a 2.2 *Kpn*I subfragment (Fig. 2b) which was also cloned in pBluescript KS to yield pBKP2.2.

**Transposon Tn5 mutagenesis.** Transposon Tn5 insertions within hybrid plasmids p2726.5 and pCOSHG5, harbouring *pcaR* and *pcaHG*, respectively, were obtained as described by Magazin *et al.* (1986) with *E. coli* HB101::Tn5 as the source of the transposon. *E. coli* HB101 cells containing Tn5 insertions within the plasmid pCOSHG5, were mated *en masse* with *P. putida* WCS358 by using helper strain *E. coli*(pRK2013) as described above. The cells from the conjugation mix were suspended and spread on LB plates containing nalidixic acid, ampicillin, tetracycline and kanamycin, selecting for strains harbouring recombinant plasmids which had received a transposon insertion. About 200 clones were grown and plasmids were purified according to Birnboim (1983). Subsequently, *E. coli* DH5 $\alpha$  was transformed and plated on LB tetracycline and kanamycin as described above. DNA was purified from transformants and the Tn5 insertions mapped by restriction analysis and DNA sequencing. One plasmid, designated pCOSHG5::Tn5 had an insertion located in the *pcaH* gene at position 300 of the submitted sequence (accession no. AJ295623) where position 111 is the ATG of *pcaH*.

*E. coli* HB101 cells containing Tn5 insertions within the plasmid p2726.5 were identified by purifying plasmid DNA from HB101::Tn5 (p2726.5) which was subsequently used to transform *E. coli* DH5 $\alpha$ . Transformants were selected for ampicillin and kanamycin resistance. Transposon insertions in the *pcaR* gene were then mapped by restriction and Southern analyses and by DNA sequencing. One Tn5 insertion in the *pcaR* gene in p2726.5 was identified (plasmid designated p27266.5::Tn5) and used in a marker exchange experiment to transfer this Tn5 insertion into the *pcaR* gene harboured in pCOS272, yielding pCOS272::Tn5. Tn5 was located at position 604 of the sequence (accession no. AJ252090) where position 135 is the ATG of *pcaR*.

**Site-specific exchange of mutations with the *Pseudomonas* chromosome.** Plasmids pCOS272::Tn5 and pCOSHG5::Tn5 carrying a Tn5 insertion in the *pcaR* and *pcaH* genes, respectively, were homogenized with the corresponding target region of the genome of *P. putida* WCS358 by a marker exchange procedure described by Corbin *et al.* (1982) and Venturi *et al.* (1998). pPH1J1 was used as the incoming IncP1 incompatible plasmid and selections were made on LB plates containing nalidixic acid, ampicillin, gentamicin and kanamycin. Putative marker-exchanged mutants were streaked on LB plates containing tetracycline to confirm loss of IncP1

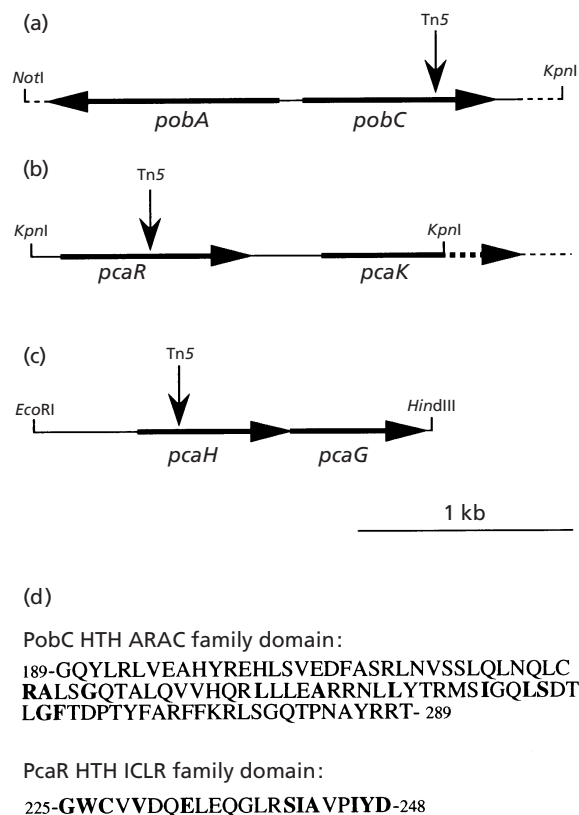
recombinant plasmids. By this method genomic Tn5 mutants were generated, one called *P. putida* VBPC, harbouring a Tn5 insertion in *pcaR*, and another designated VBHG, harbouring a Tn5 insertion in the *pcaH* gene of *P. putida* WCS358. The fidelity of each marker exchange event was confirmed by purifying chromosomal DNA of the mutants, which were analysed by digestion with restriction enzymes and via Southern hybridization, verifying that *pcaR* and *pcaH*, respectively, were mutated in the chromosome.

**DNA sequence determination and analysis.** A 2.2 kb *KpnI* fragment harbouring *pcaR* (pBKP2.2), a 4.7 kb *KpnI*-*NotI* fragment harbouring *pobA-pobC* (pB4.7KN) and a 1.8 kb *EcoRI*-*HindIII* fragment harbouring *pcaHG* (pBGH18) were subcloned from pCOS272, pCOSHB and pCOSHG5, respectively, and utilized for sequencing. The constructs were either encapsidated as single-stranded DNA upon infection with helper phage VCSM13 (Stratagene) or used directly for DNA sequencing. Several oligonucleotides were synthesized and used in sequencing reactions. Nucleotide sequences were determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) using [ $\alpha$ -<sup>32</sup>S]dATP for labelling and 7-deaza-dGTP (Pharmacia) instead of dGTP. The nucleotide sequence was determined in both directions and across all restriction sites.

## RESULTS

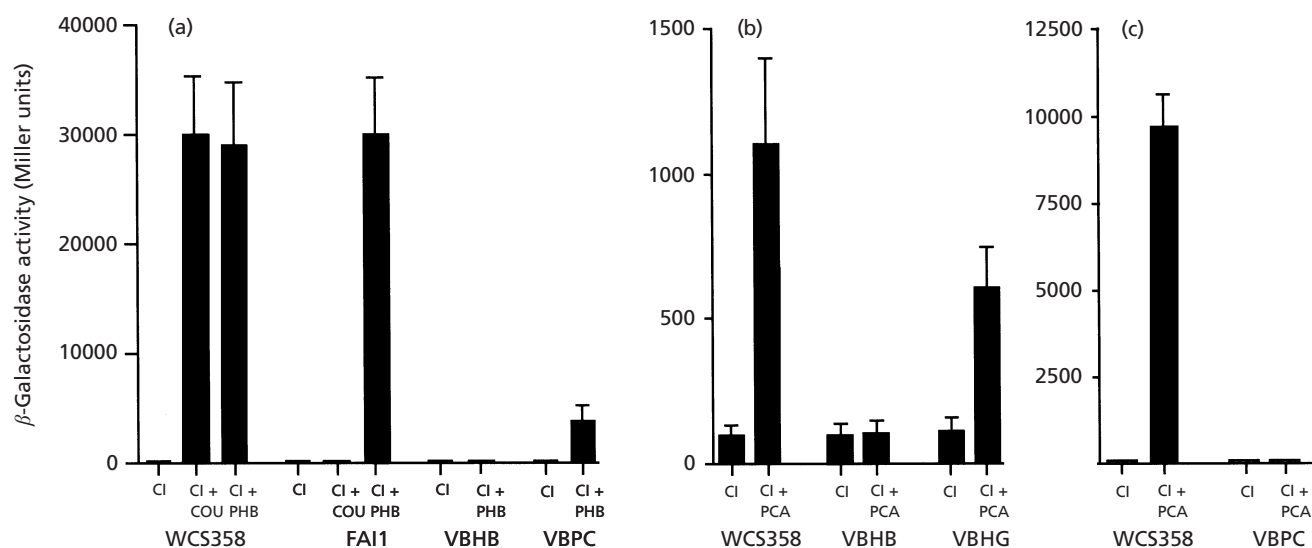
### Isolation and characterization of the *pobC-pobA* locus of *P. putida* WCS358

*P. putida* WCS358 can utilize PHB as sole carbon source. The first step in its degradation involves the conversion via hydroxylation of PHB to PCA (Fig. 1). This conversion is catalysed by *p*-hydroxybenzoate hydroxylase encoded by *pobA* (DiMarco & Ornston, 1994; Wong *et al.*, 1994). This gene is genetically linked to the *pobR* regulator which controls its expression in response to PHB. To identify the *pobR-pobA* locus of strain WCS358, 5000 genomic Tn5 mutants of strain WCS358 were screened for their ability to grow on PCA and not on PHB as sole carbon source. One mutant, designated *P. putida* VBHB, was isolated which had the desired phenotype. A cosmid genomic library of *P. putida* WCS358 was introduced in mutant VBHB and transconjugants showing complementation of this mutation were selected for growth on M9 plates containing PHB as sole carbon source. Several cosmids were purified from such transconjugants and were used separately to transform *E. coli* DH5 $\alpha$ . Subsequently, these plasmids were transferred by conjugation into mutant VBHB, confirming that they complemented the mutation for growth on PHB. Restriction analysis revealed that all hybrid cosmids contained only two *HindIII* fragments, one of approximately 25 kb and another of 3.9 kb; this complementing cosmid was designated pCOSHB. To map the complementing DNA region within pCOSHB, the DNA sequence flanking the Tn5 insertion in mutant *P. putida* VBHB was determined using an arbitrary PCR method (described in Methods). This allowed the cloning of a 400 bp fragment flanking the Tn5 insertion in mutant VBHB, the position of the transposon was found at position 249 of the submitted sequence (accession no. AJ251792) where position 991 is the ATG of *pobR*. This fragment was used as probe and allowed the



**Fig. 2.** Genetic organization of the three cloned regions harbouring the three loci. The localization of the transposon Tn5 in each mutant is indicated (see text for details). (a) *pobA-pobC*, (b) *pcaR* and *pcaK* and (c) *pcaH-pcaG*. Restriction sites used for subcloning and sequencing are shown above the three physical gene maps. (d) Prosite motifs of the two regulators; PcaR has an ICLR HTH motif (PS01051), and PobC an ARAC HTH motif (PS01124). The amino acids in bold fit the consensus pattern of the motif.

localization of the complementing region within a 4.7 kb *KpnI*-*NotI* fragment of pCOSHB which was subsequently cloned in pBlueScript KS to yield pB4.7KN. DNA sequencing of 3.3 kb of this fragment revealed the presence of the *pobA-pobC* genes of *P. putida* WCS358 (accession no. AJ251792) (Fig. 2a). The PobC protein was composed of 293 aa and had a calculated molecular mass of 33 kDa. The amino acid sequence bore an HTH AraC family (Prosite PS01124) Prosite profile between amino acids 190 and 288 (Fig. 2d). It surprisingly displayed highest identity (58%) with PobR of *Azotobacter chroococcum* and it showed only 47% identity with PobR of *P. aeruginosa* and *Pseudomonas* sp. HR199, 37% identity with PobR of *Rhizobium leguminosarum* and only 10% identity with PobR of *Acinetobacter* sp. ADP1 (data not shown). It was decided to designate the name PobC to the regulator of *pobA* identified in this study since it belonged to the AraC regulatory family and it exhibited only low identities to the PobR proteins studied thus far which belong to the ICLR family. The *p*-hydroxybenzoate hydroxylase (PobA) was composed of 396 aa and had a



**Fig. 3.**  $\beta$ -Galactosidase activities driven from the *pobA* promoter (*pobA-lacZ* fusion in hybrid plasmid pPPOBA) and from the *pcaK* promoter (*pcaK-lacZ* fusion in hybrid plasmid pPPCAK) in *P. putida* WCS358, and in various Tn5 genomic mutants. All measurements were done in triplicate and the mean value is given. Cells harbouring the plasmids were grown for 16 h in M9 medium with 0.15% citric acid and with the indicated aromatic acid added to a final concentration 0.1%. All media were supplemented with 40  $\mu$ g tetracycline ml<sup>-1</sup>. COU, *p*-Coumaric acid; Cl, citric acid. Strains: WCS358, wild-type *P. putida* WCS358 (Geels & Schippers, 1983); FAI1, *P. putida fca::Tn5* (Venturi *et al.*, 1998); VBHB, *P. putida pobC::Tn5* (this study); VBPC, *P. putida pcaR::Tn5* (this study); VBHG, *P. putida pcaH::Tn5* (this study). (a)  $\beta$ -Galactosidase activities in strains harbouring the *pobA-lacZ* fusion (pPPOBA) in response to PHB and *p*-coumaric acid. (b)  $\beta$ -Galactosidase activities in strains harbouring the *pobA-lacZ* fusion (pPPOBA) in response to PCA. (c)  $\beta$ -Galactosidase activities in strains harbouring the *pcaK-lacZ* fusion (pPPCAK) in response to PCA. See text for details.

calculated molecular mass of 44 kDa (accession no. AJ251792). It exhibited 74% identity with PobA of *Pseudomonas* sp. HR199, 70% identity with *P. fluorescens*, 60% identity with PobA of *Acinetobacter* sp. ADP1 and interestingly the highest identity (80%) was again observed with *Azotobacter chroococcum* (data not shown).

#### Gene promoter activity of the *pobA* promoter

The promoter of *pobA* was cloned upstream from a promoterless  $\beta$ -galactosidase (*lacZ*) gene in the promoter probe vector pMP220 as described in Methods. The transcriptional fusion, called pPPOBA, was conjugated into *P. putida* WCS358 and mutant derivatives and  $\beta$ -galactosidase activities were determined in response to several phenolic acids (Fig. 3).

As expected the promoter of the *pobA* gene had a strong response to the presence of PHB. When *P. putida* WCS358(pPPOBA) was grown in LB or M9 media supplemented with 0.1% PHB the promoter displayed over 30000 Miller units of  $\beta$ -galactosidase activity, indicating that it is a very strong promoter (Fig. 3a). The same induction level was also observed when the strain was exposed to *p*-coumaric acid. This was most probably due to the fact that *p*-coumaric acid is converted to PHB which would then induce the promoter as confirmed by the absence of promoter activity in the *fca::Tn5* mutant *P. putida* FAI1 (Venturi *et al.*,

1998) when exposed to *p*-coumaric acid (Fig. 3a). This mutant had a Tn5 insertion in a structural gene absolutely necessary for the conversion of *p*-coumaric to PHB. Finally, it was also observed that PCA was involved in the activation of this promoter to a lesser but significant extent (just over 1000 Miller units) (Fig. 3b).

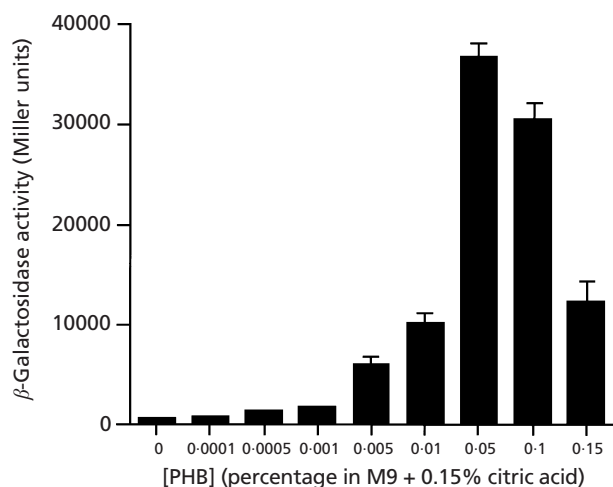
#### Phenotype of *pobC::Tn5* genomic mutants

As mentioned above, these mutants could not grow on PHB as sole carbon source but could grow on PCA, thus they were impaired only in the hydroxylation step. Gene promoter studies in the *pobC::Tn5* mutant VBHB are presented in Fig. 3. They showed that the *pobA* promoter, harboured in pPPOBA, in the parent strain was very strong and inducible by PHB, reaching a value of approximately 30000 Miller units; this activity was not observed in the *pobC* mutant VBHB (Fig. 3a). As mentioned above, activation by PCA occurred to a lesser extent in *P. putida* WCS358(pPPOBA) (1100 Miller units). This activity was not observed in the *pobC::Tn5* mutant (Fig. 3b), demonstrating that this PCA induction was occurring via PobC.

#### PCA weakly induces *pobA* gene promoter activity

It was observed that PCA weakly but significantly induces *pobA* promoter activity (see above). It was possible, however, that this induction was occurring via





**Fig. 4.**  $\beta$ -Galactosidase activities driven from the *pobA* promoter (*pobA-lacZ* fusion in hybrid plasmid pPPOBA) in response to PHB concentrations determined in *P. putida* WCS358. Activities shown are means of three independent measurements. Cells harbouring the plasmids were grown for 16 h in M9 medium with 0.15% citric acid and with the indicated concentration of PHB. All media were supplemented with 40  $\mu$ g tetracycline  $\text{ml}^{-1}$ .

another compound resulting from the stepwise transformation of PCA in the  $\beta$ -ketoadipate pathway (Nichols & Harwood, 1995; Romero-Steiner *et al.*, 1994). To establish that PCA was responsible for the observed *pobA* promoter activity we determined  $\beta$ -galactosidase levels in a *P. putida* WCS358 genomic mutant which was blocked in the first transformation step of PCA catabolism. This mutant was constructed via the cloning and characterization of the *pcaHG* locus, performing transposon mutagenesis on the cloned locus and generating a genomic mutant via a marker exchange technique (see Methods). This led to the construction of a mutant designated *P. putida* VBHG exhibiting a Tn5 insertion in the *pcaH* gene in the chromosome of *P. putida* WCS358. The *pcaHG* operon encodes two proteins (Fig. 2c) (accession no. AJ295623): PcaH (26.7 kDa), the  $\beta$ -subunit, and PcaG (22.4 kDa), the  $\alpha$ -subunit of protocatechuate 3,4-dioxygenase which transforms PCA to carboxymuconate (Nichols & Harwood, 1995; Romero-Steiner *et al.*, 1994). As expected, these two proteins have very high identities (approx. 80%) with homologues identified in other *Pseudomonas* spp. (data not shown; Overhage *et al.*, 1999a). It was verified that the *P. putida* VBHG mutant was unable to transform PCA since when present in the medium after overnight growth, the compound was not transformed. In addition this mutant could not grow in minimal medium when PCA was sole carbon source. This mutant could be complemented for these two phenotypes by pCOSHG5, carrying a 5 kb *Hind*III fragment with the *pcaHG* genes.

We determined *pobA* promoter activity in mutant *P.*

*putida* VBHG(pPPOBA) in the presence of PCA and it was observed that in this mutant the weak but significant response to PCA was retained (Fig. 3b). Thus, it was concluded that it was PCA that could act as a weak inducer of *pobA* gene expression via PobC.

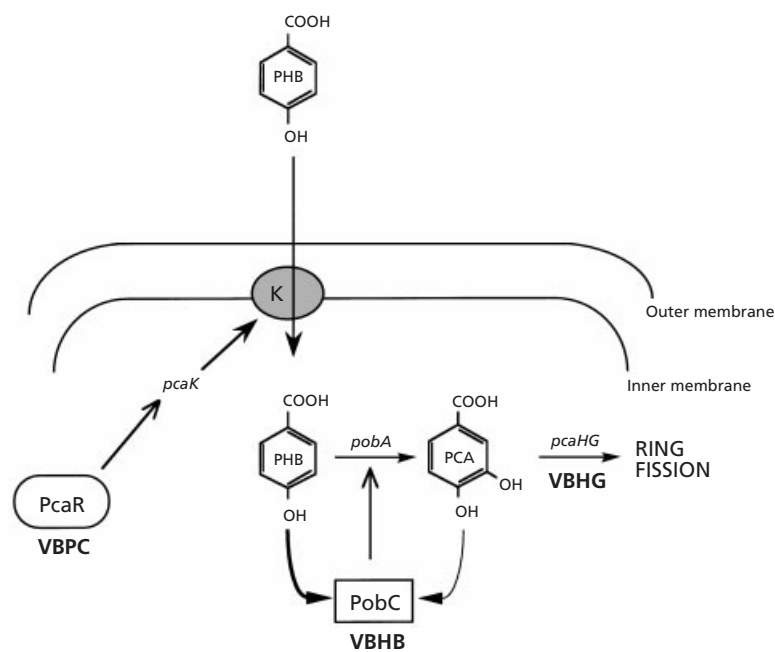
#### Expression of *pobA* is influenced by PHB concentration

The PHB-induced activity of the *pobA* promoter via PobC was determined. *pobA* promoter activity was determined in *P. putida* WCS358(pPPOBA) grown in the presence of various concentrations of PHB through  $\beta$ -galactosidase assays. As shown in Fig. 4, it was established that *pobA* gene promoter activity varied according to the amount of PHB present: the activity rose with increasing concentrations of the phenolic compound and the highest level was achieved at 0.05%. At higher concentrations the activity began to decrease and at approximately 0.15% there was a sharp drop in  $\beta$ -galactosidase activity, most probably because higher concentrations of the phenolic compound were bacteriostatic.

#### Importance of *pcaR* in *pobA* gene regulation

A regulator called PcaR has been described in *P. putida* that regulates several loci involved in the degradation of PCA. Among these genes one called *pcaK* encoding a transport protein involved in PHB transport has been identified (Nichols & Harwood, 1995; Romero-Steiner *et al.*, 1994). The regulator PcaR is required for the complete degradation of PHB; thus the possible role of this regulator in controlling gene expression of *pobA* in *P. putida* WCS358 was investigated via the identification of the gene and creation of a *pcaR*-deficient mutant. The *pcaR* gene of *P. putida* WCS358 was identified in a cosmid gene bank of strain WCS358 maintained in *E. coli* using the *pcaR* gene of *P. aeruginosa* PAO1 as probe. The gene was cloned, mutated and a *pcaR*-deficient mutant of strain WCS358 was constructed via a marker exchange technique (see accession no. AJ252090; Methods and Fig. 2b). The mutant was designated *P. putida* VBPC. As expected, this mutant was unable to grow in M9 medium containing PHB and PCA as sole source of carbon, respectively. The PcaR protein of strain WCS358 was 95% identical to PcaR of *P. putida* PRS2000, 72% identical to PcaR of *P. aeruginosa* PAO1 and 43% identical to PcaR of *Rhodococcus opacus* (data not shown). Interestingly, it also displayed 34 and 33% identity with PcaU and PobR of *Acinetobacter* sp. ADP1, respectively (data not shown). All of these proteins belong to the IclR family of bacterial regulators.

To investigate the possible role of PcaR in *pobA* gene regulation, promoter fusion pPPOBA was conjugated in mutant VBPC and activation by PHB was determined. In mutant VBPC the promoter had only 15% of promoter activity as compared to the wild-type (Fig. 3a) and it was concluded that *pcaR* played an important role



**Fig. 5.** Summary of the genetics of the regulation of *pobA* gene expression in *P. putida* WCS358. *pobA* is activated by the AraC family regulator PobC when PHB is present and to a lesser extent in the presence of PCA. The expression of *pobA* is significantly reduced in a *pcaR* mutant and it is postulated that it is due to the absence of the PHB transporter PcaK which is regulated by PcaR (see text for details).

in *pobA* gene expression. This observed effect was probably indirect via the regulation of other gene(s) important for PHB degradation. It was previously observed that in *P. putida*, *pcaR* regulates the PHB transporter gene *pcaK* (Nichols & Harwood, 1995). We observed that the same was true in strain WCS358. The *pcaK* gene was genetically linked to *pcaR* (Fig. 2b) and contained a putative *pcaR* binding region in its promoter. The *pcaK* promoter of strain WCS358 was also cloned upstream from a promoterless  $\beta$ -galactosidase gene in construct pPPCAK and this showed that in strain WCS358 this promoter was activated in the presence of PCA; this activity disappeared in the *pcaR* mutant VBPC (Fig. 3c). It was concluded that *pcaR* regulated the expression of *pcaK*; thus in a *pcaR* mutant there is less efficient transport of PHB, most probably leading to a less efficient *pobA* activation in response to PHB.

## DISCUSSION

The genetics of the regulation of the *p*-hydroxybenzoate hydroxylase *pobA* gene of *P. putida* involved in the catabolism of PHB to the central intermediate protocatechuate (Fig. 1) was studied. To our knowledge, this is only the second *pobA* regulator studied, the other being *pobA* regulation via PobR in *Acinetobacter* (Kok *et al.*, 1997). Promoter studies in strain WCS358 have shown that *pobA* gene expression was activated by PHB and, interestingly, to a lesser extent by PCA. This induction was mediated via the PobC transcriptional activator, whose corresponding gene was identified and characterized. Like all hitherto identified *pobR* genes, the *pobC* gene of *P. putida* WCS358 was in close proximity to the *pobA* structural gene. The protein of strain WCS358, however, was not so closely related to

the other PobR regulators as expected as revealed by analysis of the primary structure. PobC of *P. putida* WCS358 only exhibited identities below 50% with corresponding proteins of other *Pseudomonas* strains and only 37 and 10% identity with PobR of *R. leguminosarum* and *Acinetobacter* sp. ADP1, respectively. This difference in the primary structure most probably also reflects a functional difference. Thus the further study of this protein could be of great interest. The WCS358 PobC protein had 58% identity with the uncharacterized PobR of *Azotobacter chroococcum* and this PobR has an AraC DNA binding motif at a similar position; thus it appears that the PobR proteins belong to either the IclR or the AraC family, showing that divergent evolution has taken place. Overhage *et al.* (1999a) have depicted a relationship between the different PobR proteins identified. It appears that PobR of *Acinetobacter calcoaceticus* is evolutionarily distant from the other proteins and it is the only one studied. The designation of PobC for the protein in *P. putida* WCS358 was chosen since it belongs to the AraC family.

The *pobA* promoter was regulated by PobC and it was activated very efficiently by PHB; the levels of  $\beta$ -galactosidase activity obtained using the *pobA* promoter *lacZ* fusion indicated that this promoter is very strong, considerably stronger than the *pobA* promoter of *Acinetobacter* sp. ADP1 (DiMarco *et al.*, 1993). A weak but significant level of *pobA* promoter activity was also observed in response to PCA and this was confirmed to be most probably due to a PobC-PCA interaction since in *pobC::Tn5* mutants (VBHB) this protocatechuate-dependent activity was not observed (Fig. 3b). It was verified that it was PCA which was responsible for this weak activation and not another intermediate in its catabolism since in a *pcaH::Tn5* mutant, which cannot



further catabolize PCA, this activation was not significantly altered. To our knowledge this is the first *pobA* regulator reported to have a response activity on PCA. The co-inducer response domain of the *P. putida* WCS358 PobC, which recognizes PHB, probably also responds to protocatechuate which is very similar in structure, the only difference being a hydroxyl group at the *meta* position. This property could be helpful in future work designed to precisely define the inducer response domain via mutations which alter the response to a new effector (e.g. PCA).

The PcaR protein has been identified and characterized in *P. putida* PRS2000 as a regulator of several loci of the *ortho* cleavage pathway, e.g. *pcaBDC*, *pcaIJ*, *pcaF* and *pcaK* (Nichols & Harwood, 1995; Romero-Steiner *et al.*, 1994). The PcaR of *P. putida* WCS358 had 95% identity with PcaR of strain PRS2000 and the *pcaR*::Tn5 mutant of strain WCS358, VBPC, could no longer utilize all of the aromatic compounds shown in Fig. 1, thus it is very likely that in strain WCS358 PcaR regulates the same loci as in strain PRS2000. In strain WCS358 *pcaR* is genetically linked to *pcaK*, just like in strain PRS2000, the two genes most likely being independently transcribed. Promoter studies revealed that in the *pcaR*::Tn5 mutant there is considerable reduction in *pobA* promoter activity, displaying approximately one-sixth of the activity (Fig. 3a). This effect of PcaR on *pobA* gene expression most likely occurs indirectly. It has been demonstrated in *P. putida* PRS2000 that PcaK transports *p*-hydroxybenzoate and that *pcaK* expression is controlled by PcaR (Nichols & Harwood, 1995). In *P. putida* WCS358, just like in *P. putida* PRS2000, a *pcaR*-binding site has been observed in the *pcaK* promoter region (TGTTTCGATAAACGACAAT-247 bp-ATG; data not shown) and it was demonstrated that in *pcaR*::Tn5 mutants (VBPC) there was no *pcaK* expression (Fig. 3c). Consequently, in *pcaR*::Tn5 mutants there is less efficient transport of PHB, possibly resulting in a decrease of *pobA* promoter activity (Fig. 3a). In fact it was observed that *pobA* expression varies considerably with PHB concentration (Fig. 4). It cannot be excluded, however, that PcaR might regulate the expression of *pobC* which would result in the observed decreased *pobA* expression in the *pcaR*::Tn5 mutant. We have cloned the *pobC* promoter upstream of a promoterless *lacZ* and have observed that this promoter is weak and constitutive with respect to genetic background and presence of aromatic acids (data not shown).

This study has provided data on the regulation of *pobA* expression in *P. putida* WCS358 via PobC and PcaR, with PHB and PCA being identified as effectors. Interestingly, this demonstrated that in *P. putida* the genetically linked regulator was different in primary structure, that it responded efficiently and strongly to PHB, weakly to PCA and that PcaR was important via *pcaK* gene expression for complete *pobA* activation (Fig. 5). Future work will more precisely define the mode of action of these regulatory responses which are summarized in Fig. 5 and the role that these genes and proteins might play in signalling gene expression in the

rhizosphere in this plant-growth-promoting *Pseudomonas* strain.

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