Appl Microbiol Biotechnol (2014) 98:8267–8279
DOI 10.1007/s00253-014-5881-6

Induction and carbon catabolite repression of phenol degradation genes in *Rhodococcus erythropolis* and *Rhodococcus jostii*

Juraj Szőköl · Lenka Rucká · Michaela Šimčíková · Petr Halada · Jan Nešvera · Miroslav Pátek

Received: 9 May 2014 / Accepted: 4 June 2014 / Published online: 18 June 2014

Abstract *Rhodococcus erythropolis* CCM2595 is able to efficiently utilize phenol and other aromatic compounds. We cloned and sequenced its complete gene cluster — *catA*, *catB*, *catC*, *catR*, *pheR*, *pheA2*, *pheA1* — involved in the *ortho*-cleavage pathway of phenol. The activity of the key enzyme of the phenol degradation pathway, two-component phenol hydroxylase, was found to be induced by phenol. When both phenol and succinate were present in the medium, phenol hydroxylase activity decreased substantially. To analyze the regulation of phenol degradation at the transcriptional level, the transcriptional fusions of the divergently oriented promoters P*pheA2* and P*pheR* with the *gfp*uv reporter gene were constructed. The promoters driving expression of the genes of the *pheR–pheA2pheA1* cluster were localized by determining the respective transcriptional start points. Measurements of GFP fluorescence as well as quantitative RT-PCR revealed that expression of the *phe* genes is induced by phenol at the transcriptional level. The transcription of *pheA2A1* and *pheR* was repressed by succinate, whereas no repression by glucose or glycerol was observed. Activation of the *R. erythropolis* CCM2595 *pheA2* promoter by PheR, an AraC-type transcriptional regulator, was demonstrated by overexpression of the *pheR* gene. Analysis of the transcriptional regulation of two similar *phe* clusters from *R. jostii* RHA1 by various substrates showed that the type of carbon catabolite repression and the temporal transcriptional pattern during cultivation are different in each of the three *phe* clusters analyzed.

J. Szőköl · L. Rucká · M. Šimčíková · P. Halada · J. Nešvera · M. Pátek (✉)
Institute of Microbiology, AS CR, v. v. i., Vídeňská 1083, 14220 Prague 4, Czech Republic
e-mail: patek@biomed.cas.cz

Keywords Phenol degradation · Phenol hydroxylase · *Rhodococcus erythropolis* · *Rhodococcus jostii* · Promoter · AraC-type transcriptional regulator

Introduction

Efficient aerobic biodegradation of phenolic compounds has become an attractive alternative to physical and chemical methods to remove these toxic pollutants from the environment (Martínková et al. 2009). A number of phenol-utilizing bacterial strains were isolated and enzymes involved in the respective catabolic pathways were characterized. In the first step of phenol degradation, the aromatic ring is hydroxylated by phenol hydroxylase (PHH, phenol 2-monooxygenase EC 1.14.13.7). The catechol formed in this reaction is further degraded via an *ortho*-cleavage or *meta*-cleavage pathway to central metabolism intermediates. In many bacteria, phenol hydroxylases are the rate-limiting enzymes of phenol catabolism (Shingler 2003). Three different types of PHHs were described in terms of a number of protein subunits. Most phenol-degrading strains of the genera *Pseudomonas*, *Acinetobacter*, *Comamonas* and *Burkholderia* contain multicomponent PHHs (mPHs) encoded by six genes (Shingler 2003). Various *Pseudomonas* strains (Putrinš et al. 2007) and some Gram-positive bacteria (e.g., *Bacillus stearothermophillus*; Kim and Oriel 1995) contain a single-component PHH. Two-component flavin-dependent monooxygenases were reported in two *Bacillus* strains (Duffner and Müller 1998; Kirchner et al. 2003). A similar two-component PHH was described in *R. erythropolis* UVP-1 (Saa et al. 2009). Despite the significance of *Rhodococcus* strains as efficient phenol degraders in biotechnological processes (Paisio et al. 2012; Prieto et al. 2002), no detailed analysis of phenol catabolism and its genetic control has been performed in rhodococci so far.

Expression of the phenol degradation genes was found to be controlled in bacteria by a complex system including specific and global regulatory mechanisms (Shingler 2003). The effector compound, which is either phenol or an intermediate of its catabolic pathway (e.g., *cis*,*cis*-muconate) provides the specificity of gene expression induction. The respective protein regulators, which mediate the activation or repression of the genes, are usually encoded by genes adjacent to the catabolic genes. Expression of the *dmpKLMNOP* operon coding for mPH in *Pseudomonas* sp. CF600 is strictly controlled by the transcriptional activator DmpR (NtrC family) (Shingler et al. 1993). Genes encoding mPH in *Comamonas testosteroni* R5 were repressed by the PhcS regulator (GntR family) only in the absence of phenol, whereas this repressor did not affect the gene expression when the cells were grown on phenol. The PhcR activator (AraC/XylS family) was found to be responsible for the gratuitous expression of phenol-metabolizing genes in this strain (Teramoto et al. 2001). The first transcriptional regulator of the phenol metabolism in gram-positive bacteria was found in *Geobacillus stearothermophilus*, which degrades phenol via the *meta*-pathway; however, the mechanism of its action is still unknown (Omokoko et al. 2008). No data on the regulation of genes encoding two-component PHHs in rhodococci are available.

Expression of most of the genes involved in the catabolism of aromatic compounds including phenol is affected by the presence of alternative substrates in the growth media. Glucose, acetate or tricarboxylic acid cycle intermediates can inhibit the induction of the respective enzymes. A number of operons involved in the pathways for the catabolism of various aromatic compounds which are funneled into the β-ketoadipate pathway in *Acinetobacter baylyi* are controlled by carbon catabolite repression (CCR) mediated by succinate plus acetate. The global regulator Crc is involved in the repression of these operons (Bleichrodt et al. 2010). A negative control of phenol degradation genes by glucose, pyruvate, citrate and succinate was proved in *P. putida*. Inhibition of the activating function of the PhlR protein is involved in this regulation (Müller et al. 1996). The utilization hierarchy of carbon and energy sources may be generally considered to be a consequence of CCR although the underlying mechanisms, which are mostly unknown, may differ considerably in various bacteria.

Diverse strains of the genus *Rhodococcus* are able to degrade a wide range of aromatic compounds and persistent xenobiotics (Larkin et al. 2005; Martínková et al. 2009). These bacteria are able to survive in unfavorable conditions and possess various biotechnologically important capacities (Hernandez et al. 2008). The developed tools for gene manipulations in rhodococci currently allow bioengineers to construct new degrader strains suitable for use in biotechnological processes (van der Geize and Dijkhuizen 2004; Veselý et al. 2003).

We cloned and sequenced the gene cluster *catABC–catR–pheR–pheA2A1* involved in phenol degradation in *R. erythropolis* CCM2595. We proved that the *catABC* operon is repressed by CatR, a transcriptional regulator of the IclR family. Expression of *catR* was found to be constitutive (Veselý et al. 2007). The *R. erythropolis* CCM2595 genes of the phenol degradation gene cluster were used for the development of recombinant strains efficiently degrading phenol in phenol-containing wastewater (Zídková et al. 2013). In the present work, we analyzed expression of the genes *pheA2A1*, encoding a two-component PHH, and *pheR*, encoding an AraC-type transcriptional regulator, that are involved in the first step of phenol degradation in *R. erythropolis* CCM2595 and *R. jostii* RHA1.To our knowledge, this is the first analysis of the transcriptional regulation of genes encoding two-component phenol hydroxylase.

Materials and methods

Strains, plasmids and growth conditions

The strains *Rhodococcus erythropolis* CCM2595 (Veselý et al. 2003) and *R. jostii* RHA1 (McLeod et al. 2006) were the sources of phenol degradation genes and regulatory *pheR–pheA2* sequences. *Corynebacterium glutamicum* RES167 (Dusch et al. 1999) was used as a heterologous host lacking the PheR regulator. *Escherichia coli* DH5α (Hanahan 1985) was used for cloning. The plasmids used are listed in Table 1. *Rhodococcus* strains were cultivated at 25 °C in complete 2xYT medium (Sambrook and Russel 2001) or in minimal BSM basal salt medium (Veselý et al. 2007) with various substrates: phenol (3.5 mM), protocatechuic acid (3.5 mM), benzoate (3.5 mM), *p*-hydroxybenzoate (3.5 mM), glucose (10 mM), succinate (12 mM) and glycerol (20 mM). *E. coli* was grown in LB medium at 37 °C. When appropriate, kanamycin was added to the media for *E. coli* (30 μg/ml), *C. glutamicum* (30 μg/ml) and *Rhodococcus* (200 μg/ml) and tetracycline to the media for *C. glutamicum* (10 μg/ml).

DNA techniques

DNA isolation from *E. coli*, transformation of *E. coli*, polymerase chain reaction (PCR), DNA cloning and DNA analysis were performed following standard procedures (Sambrook and Russel 2001). Plasmid DNA from *Rhodococcus* strains was isolated as described previously (Veselý et al. 2007) and transformation of *R. erythropolis*, *R. jostii* and *C. glutamicum* was done by electroporation (Veselý et al. 2003).

Table 1 Plasmids

Plasmid	Relevant characteristics	Reference
pSRK21	*E. coli–R. erythropolis* cloning vector, Km^R	(Veselý et al. 2003)
pEPR1	*E. coli–Rhodococcus–Corynebacterium* promoter-test vector, Km^R promoter-less *gfp*uv as a reporter	(Knoppová et al. 2007)
pEC-XT99A	*E. coli–C. glutamicum* expression vector, P*trc*, Tc^R	(Kirchner and Tauch 2003)
pKSAC45	*E. coli* vector for chromosomal integrations, Km^R	(Holátko et al. 2009)
pSRK*pheRA2A1*	pSRK21 carrying the *pheRA2A1* cluster	This work
pEPRP$pheA2_{Re}$	pEPR1 carrying the *R. erythropolis pheA2* promoter	This work
pEPRP$pheR_{Re}$	pEPR1 carrying the *R. erythropolis pheR* promoter	This work
pEPRP$pheA2_{RjI}$	pEPR1 carrying the *R. jostii pheA2* I promoter	This work
pEPRP$pheR_{RjI}$	pEPR1 carrying the *R. jostii pheR* I promoter	This work
pEPRP$pheA2_{RjII}$	pEPR1 carrying the *R. jostii pheA2* II promoter	This work
pEPRP$pheR_{RjII}$	pEPR1 carrying the *R. jostii pheR* II promoter	This work
pEC-XT99A*pheR*	pEC-XT99A carrying the *pheR* gene	This work
pEPRP*pheA2*WT	pEPR1 carrying the *R. erythropolis pheA2* promoter	This work
pEPRP*pheA2*M8	pEPR1 carrying the mutated *R. erythropolis pheA2* promoter	This work

RNA isolation and primer extension analysis

R. erythropolis CCM2595 and *R. jostii* RHA1 cells were cultivated in the BSM medium with phenol (3.5 mM) at 25 °C, harvested at OD_{600}=1 (16 to 18 h), washed with PBS buffer and frozen at −70 °C. The cells were disintegrated in FastPrep FP120 (BIO101) (6×20 s, speed 6.0) using glass beads (size 10). The cell debris was removed by centrifugation and total RNA was isolated from 4 ml of extract using the High Pure RNA Isolation Kit (Roche). Primer extension analysis was done as described previously (Pátek et al. 2003). Reverse transcription was carried out using 50–60 μg of RNA, SuperScript III transcriptase (Invitrogen) and the fluorescein-labeled primer GFP1 (CTAATTCAACAAGAAT TGGGAC) or GFP5 (GTTCTTCTCCTTTACTCATTT) complementary to the *gfp*uv sequence in the vector pEPR1. The reverse transcript was run on polyacrylamide gel electrophoresis in an automatic ALF DNA Sequencer (Pharmacia Biotech) with the DNA sequencing reactions generated using the same labeled primer.

Quantitative real-time RT-PCR

Total RNA was treated with TURBO DNase (Ambion) to remove DNA contamination and purified with the RNeasy kit (Qiagen). Reverse transcription was performed with SuperScript III Reverse Transcriptase (Invitrogen) in a 20-μl reaction mixture containing 2 μg of total RNA, 4 μl 5× First-Strand buffer, 1 μl dNTP mix (10 mM), 1 μl random hexamer primers (100 ng/μl) and 1 μl RNase OUT Recombinant Ribonuclease Inhibitor (40 U/μl). The reaction was run for 1 h at 50 °C and was stopped by heat inactivation of the enzyme (15 min at 70 °C).

Real-time PCR was carried out in a 25-μl reaction volume containing 2 μl cDNA,1 μl of each of the forward and reverse primers (400 nM), 12.5 μl 2× SYBR Green Supermix (Bio-Rad) in iQ5 Thermocycler (Bio-Rad). Each reaction was done in triplicate. The primers used are listed in Table 2. Cycling conditions: predenaturation at 95 °C for 5 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 20 s and elongation at 72 °C for 20 s. After qPCR completion, melting curves were generated between 55 °C and 95 °C with 0.5 °C increments.

The $\Delta\Delta C_t$ method was used for calculating the amount of transcripts with *dinB* (coding for DNA polymerase IV in *R. erythropolis* CCM2595) as a reference gene. The relative abundance of transcripts was calculated by normalization to *dinB* mRNA. Relative gene expression was reported as the change (*n*-fold) determined from the mean normalized expression relative to the mean normalized expression of the reference gene ($\Delta\Delta C_t$ method).

Phenol hydroxylase assay and phenol concentration measurements

R. erythropolis cells grown in minimal BSM medium with various substrates were disrupted with the FastPrep FP120 homogenizer (BIO101) (6×20 s, speed 6.0). Phenol hydroxylase activity (phenol monooxygenase, EC 1.14.13.7) was assayed spectrophotometrically at 340 nm in the 1.5-ml reaction mixture containing cell extract (0.05–0.1 mg protein), potassium phosphate buffer (0.1 mM, pH 7.6), 50 μl NADPH (5 mM), and 50 μl phenol (5 mM). One enzyme unit was defined as the amount of enzyme which causes the oxidation of 1 μmol NADPH/min in the presence of phenol (Neujahr

Table 2 Oligonucleotides used as PCR primers for cloning the *pheR* gene, *pheA2* promoter and *pheR–pheA2* intergenic regions and for quantitative RT-PCR (5′–3′ sequence)

Gene/region	Forward	Reverse	Amplicon size (bp)
Cloning			
pheR-A2$_{Re}$	AAGGATCCTCTAGAGTTGGTGCAGGTGAT	AACTGCAGGATCCGAAACCGCATCACAC	434
pheR-A2$_{RjI}$	CGCTTCGTCCCAATCTTCG	GAGTTCGCGTTGCTCCAT	473
pheR-A2$_{RjII}$	TCGTGAACCTCGTCCCAGTCG	TGGCCGAAAATGTTACGCAGAG	561
pheR$_{Re}$	TCGAATTCGGCAATGTGGCTGGTC	AACTCTAGACAGACATGGGATACC	1002
RT-PCR			
catA	TGCCTGCCAAGTGTGAA	GGAGTAGTAGCCCTCGTCGT	146
catB	GCAGGCTACTACCGATTCC	TGGTCTTGAGGGCGATG	108
catC	CTTCAGCGCCAGGGAAAG	ACAGGGGCAGGTTCCAGAG	121
catR	GAAGTGCTGCCCGATACTCA	ATCCTCCTCACGCTCCAGAA	93
pheA1	ATGGACGAGAACGACACCA	AAACCCGATTGAGGGAAGAA	113
pheA2	CGAAGCACTTTTCACGACCT	GTGCGGGCTGAAGATGAA	127
pheR	CAATGCCCGAAAGTGAGG	CATCTCTGCTGCGGTCCA	110
dinB	GACTCCGGCCAACTCCAC	GGATGTCGTTGTATTCGGTTC	122

and Gaal 1973). Specific activity was calculated as enzyme units per mg protein. Protein concentration was determined using the method of Bradford (1972).

Phenol concentration in the medium was measured using the colorimetric method (Martin 1949). Phenol reacts with 4-aminoantipyrine under alkaline conditions to form a red indophenol dye, which is assayed spectrophotometrically at 502 nm.

Construction and use of the two-plasmid system in *C. glutamicum*

The *R. erythropolis* CCM2595 *pheR* gene was amplified by PCR and cloned in the expression vector pEC-XT99A. The promoters P*pheA2*$_{Re}$ and P*pheA2*$_{Re}$M8 (carrying 8 nt alterations upstream of the core promoter sequence as shown in Fig. 3) were cloned in parallel in promoter-test vector pEPR1 as 100-bp fragments (10 nt downstream and 89 nt upstream of TSP) synthetized by Sigma-Aldrich. The two-plasmid strains, *C. glutamicum* (pEC-XT99A*pheR*+pEPRP*pheA2*WT) and *C. glutamicum* (pEC-XT99A*pheR*+pEPRP*pheA2*M8), were cultivated in 2xYT medium at 30 °C with shaking, isopropyl-β-thiogalactoside (IPTG) was added (0.5 mM) at OD_{600}=0.5 and cultivation continued at 25 °C for 2 h.

Promoter activity measurements

Activity of the promoters cloned in the promoter-test vector pEPR1 was quantified as the fluorescence of the cell suspension with the OD_{600}=0.2 in arbitrary units (AU) measured with a Saphire2 microplate spectrophotometer (Tecan; excitation wavelength, 395 nm; emission wavelength, 509 nm) as described previously (Knoppová et al. 2007).

Results

Cloning and sequencing of the *phe–cat* gene cluster from *R. erythropolis* CCM2595

Analysis of the *catR–catABC* gene cluster from *R. erythropolis* CCM2595 showed that the genes encode the enzymes of a part of the phenol degradation pathway, which successively convert catechol into muconolactone via an *ortho*-cleavage pathway (Veselý et al. 2007). To find if the flanking regions encode the enzymes of the upstream or downstream part of the degradative pathway, we cloned these regions using the plasmid rescue technique (Veselý et al. 2007). Fragments flanking the *catRABC* cluster were sequenced. A cluster of three genes was detected downstream of the *catR* gene (Fig. 1). Two genes were found to encode proteins homologous to the subunits of two-component PHH and were therefore designated *pheA1* and *pheA2*. The third gene coding for a transcriptional regulator homologous to AraC-type activators was designated *pheR*. The deduced amino acid sequences of PheA1 (large subunit of PHH, monooxygenase component) and PheA2 (small subunit of PHH, flavin reductase component) gene products exhibited a high degree of identity with the homologous potential proteins from *R. erythropolis* PR4 (99 % and 100 %, respectively) and lower similarity to the deduced PheA1 and PheA2 proteins from a few other *Rhodococcus* strains. Two similar clusters *pheR–pheA2A1* (I and II) were found in the genome of *R. jostii*

RHA1. These genes are not adjacent to the *cat* cluster as in *R. erythropolis*. The deduced *R. jostii* RHA1 PheA1 and PheA2 proteins exhibited 76 % to 89 % amino acid identity to their *R. erythropolis* CCM2595 homologs. The deduced amino acid sequence of the *pheR* product from *R. erythropolis* CCM2595 exhibited 99 % sequence identity with the putative PheR protein of *R. erythropolis* PR4. The two putative PheR proteins of *R. jostii* RHA1 showed lower similarities (54 % and 62 % identical amino acid residues, respectively) to their *R. erythropolis* CCM2595 homolog.

The structure of the complete cluster of phenol degradation genes from *R. erythropolis* CCM2595 is shown in the upper part of Fig. 1. The respective DNA sequence (6,967 bp) was deposited in the GenBank under Acc. No. FM995530.

Activity of phenol hydroxylase

To prove that the *pheA2* and *pheA1* genes are responsible for the PHH activity of *R. erythropolis* CCM2595, we constructed a knock-out mutant by insertional inactivation. The *pheA2A1* internal fragment (832 bp) was cloned in the *Eco*RI site of the *E. coli* vector pKSAC45 and the resulting construct was transferred to *R. erythropolis* CCM2595 by electrotransformation. Clones with the plasmid inserted into the chromosome by homologous recombination were selected on plates with kanamycin. The disruption of the *pheA2A1* genes was confirmed by PCR. The *pheA1A2* knock-out strain grew in BSM with succinate or glucose but not in the medium with phenol as the only carbon and energy source. This result indicated that the *pheA1A2* genes encode the only phenol-degrading enzyme in *R. erythropolis* CCM2595. PHH activity was determined in the extract of the wild-type cells growing on phenol and on the mixture of phenol+protocatechuate. The activity levels were almost identical (0.55±0.13 and 0.56± 0.08 U/mg protein). In contrast, PHH activity in extracts of the *R. erythropolis pheA2A1* knock-out mutant cultivated on phenol+protocatechuate were lower than 0.01 U/mg protein. These results confirmed that the *pheA1* and *pheA2* genes encode components of PHH. To overexpress these genes in the natural host cells, a 3.5-kb fragment carrying *pheA2A1* and

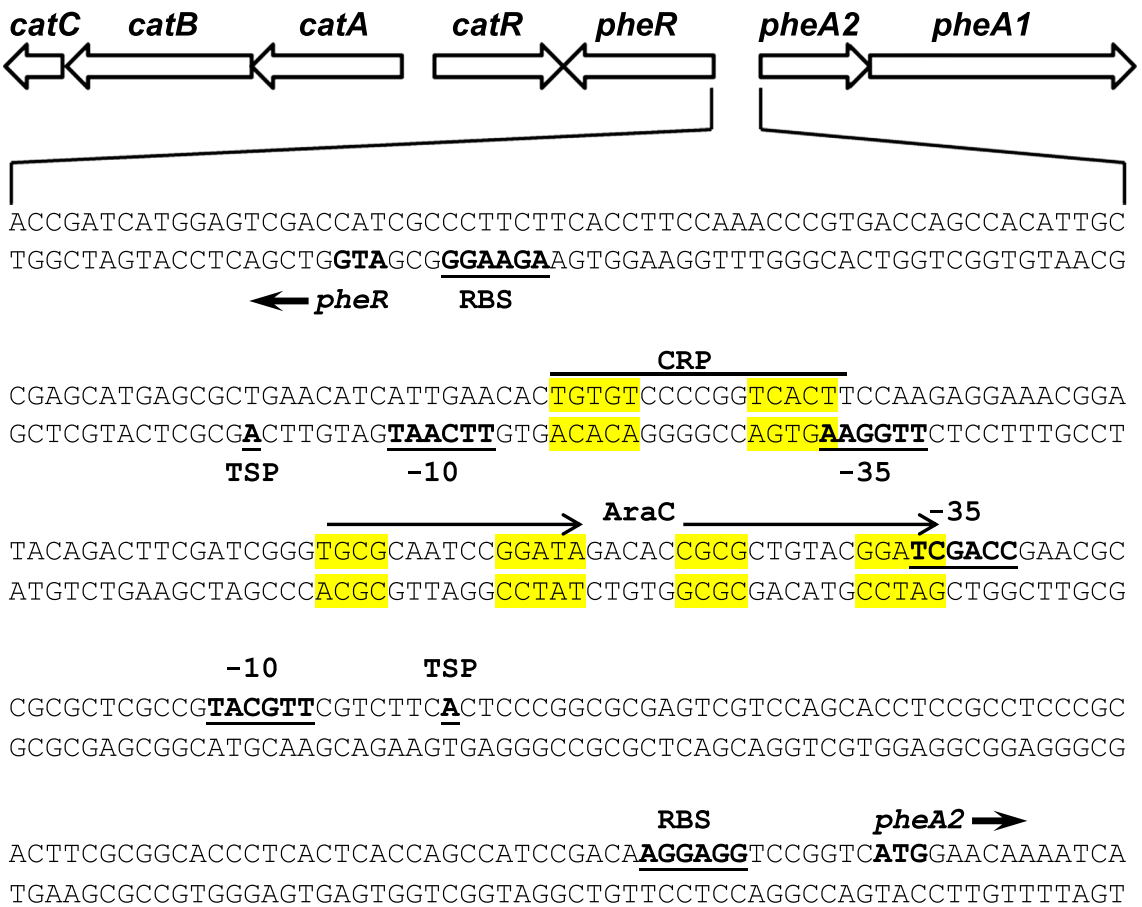

Fig. 1 Scheme of *phe–cat* gene cluster of *R. erythropolis* CCM2595 and sequence of the *pheR–pheA2* intergenic region with proposed regulatory elements. Experimentally determined transcriptional start points (*TSP*) and the proposed –10 and –35 promoter regions and ribosome-binding sites (*RBS*) are in *bold* and *underlined*. The proposed motifs of the binding sites for the CRP-type protein and the tandem binding sites for the AraC-type regulator are *shaded*, imperfect direct repeats are indicated by *arrows*. The organization of the *catR–catA* intergenic region responsible for the regulation of *catABC* expression has been described previously (Veselý et al. 2007)

pheR was cloned in the *Sma*I site of the multicopy *E. coli–Rhodococcus* vector pSRK21. PHH activity in the extracts of *R. erythropolis* cells carrying the resulting construct, pSRK*pheRA2A1*, cultivated on phenol reached 0.86±0.2 U/mg protein.

Likewise, the activity of catechol 1,2-dioxygenase encoded by the *catA* gene in *R. erythropolis* CCM2595 (Veselý et al. 2007), PHH activity was induced by phenol in this strain. Specific PHH activity in the extracts of cells cultivated on glucose or succinate was very low, whereas the activity increased 14- to 28-fold on phenol (Table 3). To find out if some utilized substrates repress the induction of PHH, we cultivated *R. erythropolis* CCM2595 in the presence of phenol and an additional substrate (glucose, succinate or protocatechuate). The presence of protocatechuate did not affect the induction of PHH by phenol. When both phenol and glucose were present in the medium, PHH activity was 64 % of that with phenol alone and the addition of succinate to the medium with phenol resulted in much larger decrease of activity (18 % of that with phenol alone) (Table 3). These data indicate that some type of partial CCR controls the utilization of phenol in *R. erythropolis* CCM2595.

Activity of the P*pheA2* and P*pheR* promoters from *R. erythropolis* and *R. jostii*

To study the transcription of the *R. erythropolis* CCM2595 *pheA2A1* and *pheR* genes, transcriptional fusions of the respective promoters (P$pheA2_{Re}$ and P$pheR_{Re}$) with the *gfp*uv reporter gene were constructed. The 434-bp DNA fragment of the intergenic region *pheR–pheA2* (including 62 and 72 bp of the *pheR* and *pheA2* 5′-ends, respectively) amplified by PCR was cloned in both orientations in the promoter-test vector pEPR1 carrying the *gfp*uv reporter gene. The green fluorescence of the *R. erythropolis* colonies carrying the resulting constructs pEPRP$pheA2_{Re}$ and pEPRP$pheR_{Re}$ on the plates with phenol indicated that divergent promoters resided on the fragment. To localize the promoters precisely, the transcriptional start points (TSPs) of *pheA2* and *pheR* were determined by primer extension (Fig. 2). The TSPs were detected 86 and 55 nt upstream of the translation initiation codons of *pheA2* and *pheR*, respectively (Fig. 1, lower part). Potential –35 and –10 promoter hexamers were recognized closely upstream of the TSPs (Fig. 1).

Table 3 Phenol hydroxylase activity in extracts of *R. erythropolis* CCM2595 cells grown on various substrates and their mixtures

Substrate	Specific activity±SD[a] (U/mg protein)
Phenol	0.55±0.130
Glucose	0.02±0.009
Succinate	0.04±0.006
Phenol+glucose	0.35±0.090
Phenol+succinate	0.10±0.006
Phenol+protocatechuate	0.56±0.080

[a] Standard deviation (SD) was calculated from three independent measurements

To compare the transcriptional control of the *phe* genes from *R. erythropolis* with the control of the similar *phe* genes from *R. jostii*, we also cloned the *pheR–pheA2* intergenic regions of the two *phe* clusters (I and II) from *R. jostii* RHA1. The resulting promoter-carrying plasmid constructs pEPRP$pheA2_{RjI}$, pEPRP$pheR_{RjI}$, pEPRP$pheA2_{RjII}$, and pEPRP$pheR_{RjII}$ were used for the same analyses as those with *R. erythropolis* promoters. The TSPs of three genes (both *pheA2* and *pheR* of cluster II and *pheA2* of cluster I) were determined by primer extension (data not shown), whereas the TSP of *pheR* of cluster I could not be detected. The TSPs are shown in Fig. 3. The high level of similarity between deduced –35 and –10 motifs of *R. erythropolis* and *R. jostii* P*pheA2* promoters is evident in Fig. 3.

To test whether the induction of PHH is controlled at the transcriptional level, the activity of the promoters P$pheA2_{Re}$ and P$pheR_{Re}$ in transcriptional fusion with *gfp*uv during growth on various substrates was determined by measuring the green fluorescence of the *R. erythropolis* cells. The fluorescence levels measured for the cells with empty vector pEPR1 (negative control) vary between 0.2 and 0.3 AU. Levels of transcription from P$pheA2_{Re}$ close to the negative control were detected in cells grown on BSM with glucose, glycerol or succinate as the sole carbon source (Fig. 4a). In contrast, cells with P$pheR_{Re}$ exhibited 2- to 3-fold higher fluorescence when grown on succinate or glycerol (Fig. 4b). This indicated that there was a certain level of constitutive activity of P$pheR_{Re}$. In agreement with this finding, *R. erythropolis* colonies carrying pEPRP$pheR_{Re}$ exhibited green fluorescence when grown on complete medium without phenol whereas the colonies with pEPRP$pheA2_{Re}$ did not. The activity of both promoters was strongly induced by phenol (Fig. 4a, b).

To examine the possible repression of phenol induction by other carbon sources, promoter activities were measured in cells grown in media containing phenol and an additional substrate. Transcription from both promoters was not decreased by the presence of glucose and glycerol in the medium with phenol. In contrast, the activity of P$pheR_{Re}$ was significantly decreased on phenol+succinate and was close to the activity of the promoter on succinate alone. The activity of P$pheA2_{Re}$ on phenol+succinate was approximately 60 % of the activity on phenol alone for the first 18 h of cultivation (Fig. 4a). The transcription from both P$pheA2_{Re}$ and P$pheR_{Re}$ increased in the later stages of cultivation (after 24 h), when the succinate was exhausted. We can conclude that there is a low-level constitutive transcription from P$pheR_{Re}$, which is

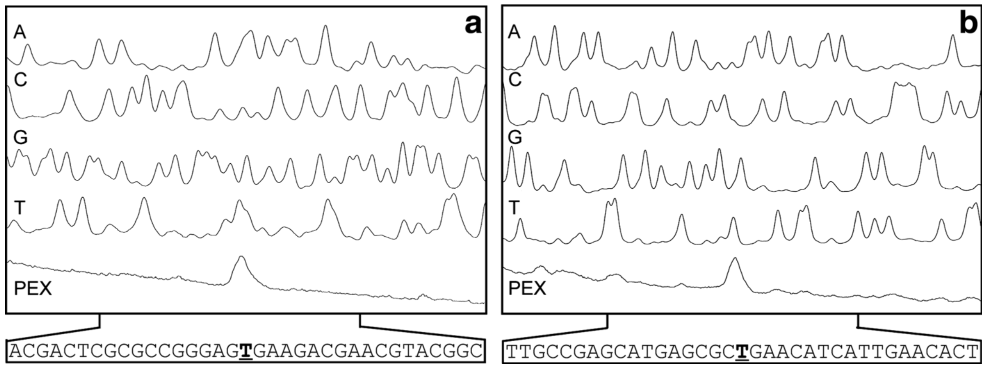

Fig. 2 Determination of *R. erythropolis* CCM2595 *pheA2* and *pheR* transcription start points by non-radioactive primer extension (PEX) analysis. **a** *pheA2*; **b** *pheR*. The bottom peaks (*PEX*) represent cDNAs synthesized in reverse transcription (primer extension) with RNA from *R. erythropolis* CCM2595. The peaks in lanes *A*, *C*, *G*, *T* represent the products of the sequencing reactions carried out with the same fluorescein-labeled primer as that used for primer extension. TSP within the relevant part of the non-coding strand sequence shown below is in *bold* and *underlined*

strongly induced by phenol. P*pheA2*$_{Re}$ activity is completely induced by phenol.

Promoter activity measurements using *gfp*uv reporter assay with promoters of the genes *pheR* I and II and *pheA2* I and II from *R. jostii* showed that the trends of activity of *pheA2* promoters during 24-h cultivation were similar to those of the respective *pheR* promoters (Fig. 4c–f). No transcription from the P*pheA2*$_{Rj\mathrm{I}}$ and P*pheR*$_{Rj\mathrm{I}}$ promoters was detected on glucose, glycerol or succinate, whereas activity was moderately induced by phenol. Phenol-induced activity was low but not repressed by any additional substrate tested (Fig. 4c, d). The transcriptional pattern found for the promoters of the *phe* II *R. jostii* cluster was different from those of cluster I promoters. Phenol induction of both P*pheA2*$_{Rj\mathrm{II}}$ and P*pheR*$_{Rj\mathrm{II}}$ was partially repressed by both glucose and glycerol, whereas strong repression of both promoters was observed when succinate was added to the medium with phenol (Fig. 4e, f).

Taken together, these results indicate that although the principle of the transcriptional regulation of the three analyzed *phe* gene clusters (one from *R. erythropolis* CCM 2595 and two from *R. jostii* RHA1) is the same (phenol induction), the phenotypic effects of various substrates differ.

In some previously described phenol degradation operons, the presence of other aromatic substrates in the growth medium affected the induction of catabolic genes (Putrinš et al. 2007). We therefore tested the influence of benzoate, *p*-hydroxybenzoate and protocatechuate on the activity of the P*pheA2*$_{Re}$ and P*pheR*$_{Re}$ promoters. The activity of neither of the two promoters was induced when any of these compounds was used as the sole carbon source. None of these aromatic substrates affected transcription from either of the two promoters when present in the medium with phenol (data not shown).

```
                          ←pheR         TSP    -10              CRP       -35          ↓
R.erythropolis CCM2595    ACCAT-46 nt-ATGAGCGCTGAACATCATTGAACACTGTGTCCCCGGTCACTTCCAAGAGGAAAC-GGATACAGACTTCGATCGGG
R.jostii RHA1 (I)         CCCAC-37 nt-TTGGACGCGGGCAGCATGGCACCAGTGTGACTACGGACACCTTCGATTGCAATT-TTCGGCGTGATCCAAGTCAC
R.jostii RHA1 (II)        CGCAT-42 nt-GGCAACTCCCGACCCTAGCATCGCCTGTGACTCACGTCACTGCAAGGGCGTCCGACCCCTGTCCGGCAAACCCGT
R.opacus B4 (I)           GACAC-62 nt-CAGTGTGACCGCGACATGGCACCAGTGTGACCGCGCACACTCCGCAATGCAATCGAGGCCCCCGCCCCAACTCGC
R.ruber BKS 20-38 (I)     ACCAC-109 nt-GTGTTCGCGAACCCTACTACCCAGTGTGATGGGCAACACGTCCCCGGGCAAACAGCGTCGTTCGTCACGACGAC
Nocardia sp. C-14-1       TGCAT-138 nt-GTGTCCGCGAACACTACTACCCAGTGTGATGAGCAACACGTCCCCGGGCAAACAGCGTCGTTCGTCATGACGAC

                          ──────────────→ AraC ──────────────→-35                -10       TSP      pheA2→
R.erythropolis CCM2595    TGCGCAATCCGGATAGACACCGCGCTGTACGGATCGACCGAACGCCGCGCTCGCCGTACGTTCGTCTTCAC-84 nt-ATGGAA
R.jostii RHA1 (I)         CGCGCAAAGCGGATCACCTCCGCGCTCAGCGGATCGACGCACCGTTCCCACGGTCGTACTTTCGTCCGTAT-37 nt-ATGGAG
R.jostii RHA1 (II)        TTCGCGTTCCGGATCCCGCCTTCGCAATGCGGATCGACGGACTGTGACGGCCGTCATACGTTGTTCAGGTC-51 nt-ATGGAC
R.opacus B4 (I)           CGCGCAAAGCGGATCACCTCCGCGCTCAGCGGATCGACGCCCCGTTCCCCCGGTCGTACGTTCGTCCGTCC-35 nt-ATGGAG
R.ruber BKS 20-38(I)      CGCGTCGAACGGATAGCGACCGCGCTCAGCGGATCGCGAAGGCCGTCCGGCCGCTCCTATGGTCTACATCA-34 nt-ATGGAA
Nocardia sp. C-14-1       CGCGTCGAACGGATAGCGACCGCGCTCAGCGGATCGCGAAGGCCGTCCGACCGCTCCTATGGTCTACATCA-34 nt-ATGGAT

nt alterations M8 in                TCCG      AAGC
R.erythropolis CCM2595
sequence
```

Fig. 3 Alignment of putative regulatory sequences within homologous *pheR–pheA2* intergenic regions of *R. erythropolis* CCM2595 and related strains. Experimentally determined transcriptional start points (*TSP*) and proposed –35 and –10 promoter motifs are in *bold* and *underlined*, the proposed motifs of the binding sites for the CRP-type protein and the tandem binding sites for the AraC-type regulator are *shaded*, imperfect direct repeats are indicated by *arrows*. The 5′ end of the 100-bp fragment used for testing the activation of the *pheA2*$_{Re}$ promoter by PheR is marked by *vertical arrow*. Nucleotide alterations introduced into the *R. erythropolis* CCM2595 P*pheA2* promoter (resulting in P*pheA2*M8) are shown below the sequences. GenBank accession numbers of the sequences: *R. erythropolis* CCM2595 (FM995530), *R. jostii* RHA1 (NC_008268), *R. opacus* B4 (NC_012522), *R. ruber* BKS 20-38 (AOEX01000015), *Nocardia* sp. C-14-1 (DQ267826)

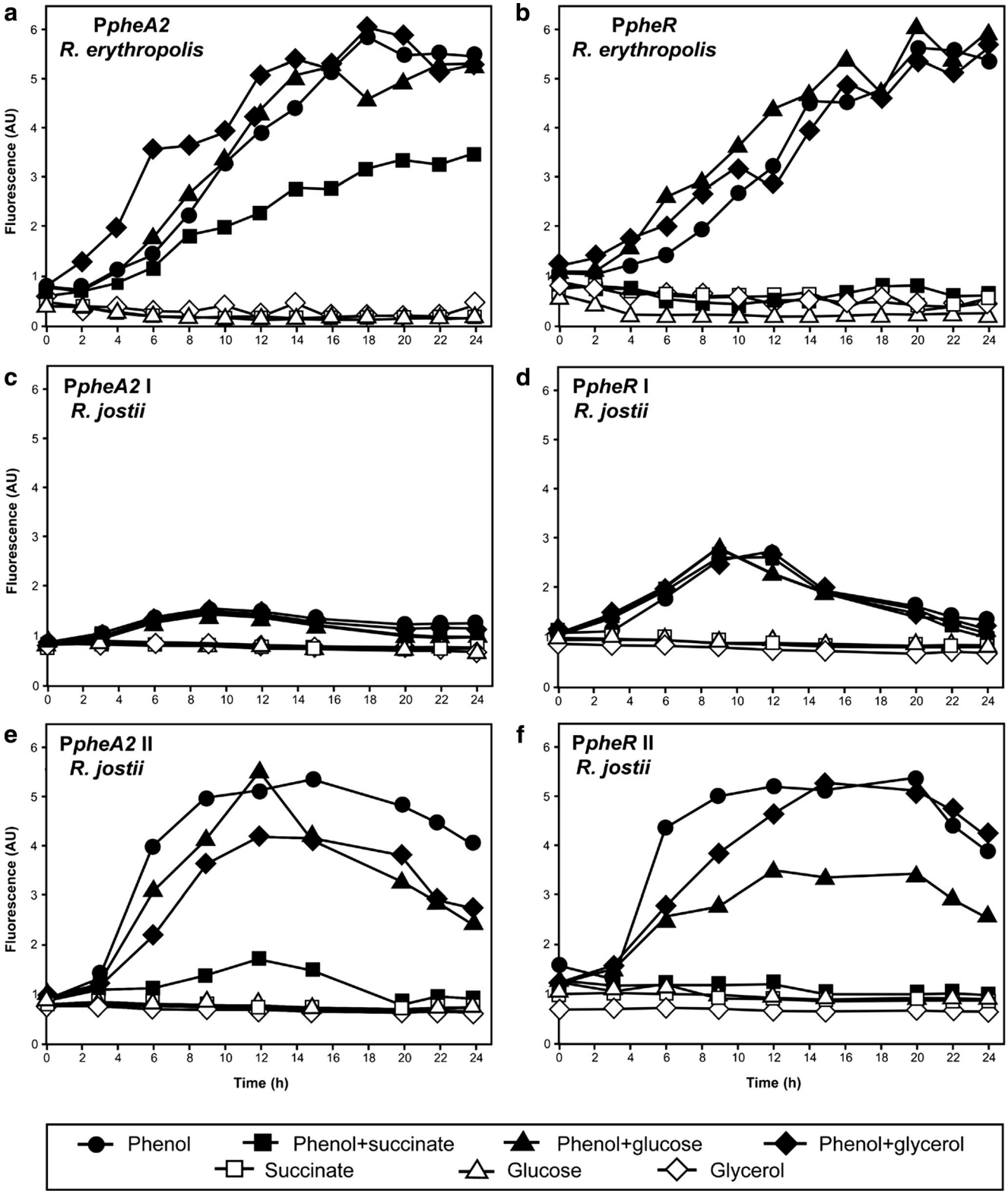

Fig. 4 Activity of *PpheA2* and *PpheR* promoters from *R. erythropolis* CCM2595 and *R. jostii* RHA1 (promoters of clusters *pheRA2A1* I and II) during growth on minimal medium BSM with various substrates. Activity was determined by measuring the intensity of fluorescence of the GFPuv reporter. *AU* arbitrary units. The values for the negative control (empty vector pEPR1) were 0.25±0.05. The values shown are averages of three independent biological replicates; standard deviations were within 15 %

Induction of *R. erythropolis* CCM2595 *phe* genes by low phenol concentration

To find the lowest concentration of phenol that can induce the transcription of *phe* genes, we determined the activity of the P*pheA2*$_{Re}$ and P*pheR*$_{Re}$ promoters using *R. erythropolis* cultures grown in BSM with various phenol concentrations (1.2, 0.6, 0.12, 0.06, 0.012 mM). The lowest phenol concentration at which a promoter activity was detected was 0.12 mM (data not shown). Cultivation on 1.2 mM phenol resulted in lower maximal P*pheA2*$_{Re}$ and P*pheR*$_{Re}$ activities (52 % and 63 %, respectively) than on 3.5 mM phenol (Fig. 5). The activity of both promoters quantitatively decreased with decreasing phenol concentration (data not shown). This indicates that expression of *phe* genes is induced by phenol in a dose-dependent manner rather than by an on/off mechanism. The maximum response of P*pheR*$_{Re}$ activity was achieved after 4 h of cultivation, whereas the maximum of P*pheA2*$_{Re}$activity was detected 6 h later (Fig. 5). The observed delayed activation of P*pheA2*$_{Re}$ transcription in comparison with that of P*pheR*$_{Re}$ transcription is in agreement with the assumption that PheR acts as an activator of *pheA2* transcription.

Quantification of the *phe* and *cat* transcripts by real-time RT-PCR

To confirm the results of promoter activity measurements using transcriptional fusion with *gfp*uv, we quantified the levels of *cat* and *phe* transcripts from *R. erythropolis* CCM2595 using quantitative real-time RT-PCR (qPCR). The lowest levels of the transcripts were found in cells grown on succinate, and these values were used as a basal level for comparison with the quantities determined on phenol. The levels of *catA*, *catB* and *catC* transcripts were approximately 60-fold higher on phenol than on succinate, whereas *catR* transcript was not induced by phenol and its level was similar on phenol and succinate (Fig. 6). These values are in good agreement with *catA* and *catR* promoter activities determined previously (Veselý et al. 2007). The *pheA1* and *pheA2* transcripts were upregulated to the same level in cells grown on phenol, which indicates that these genes form an operon. The *pheR* transcript increased much less, which probably reflects its partial constitutive expression.

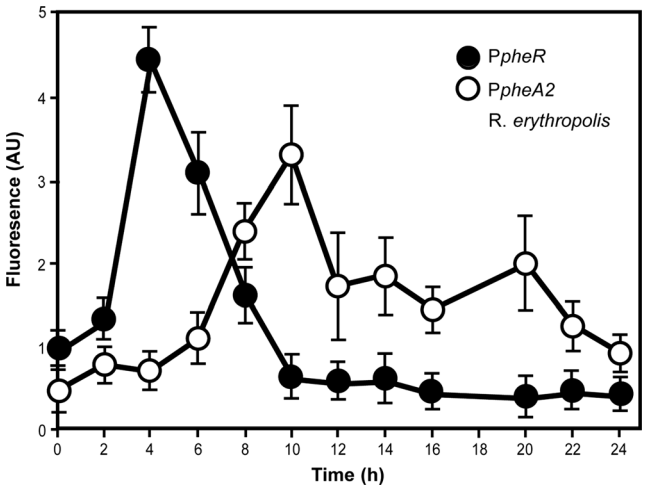

Fig. 5 Activity of P*pheA2*$_{Re}$ and P*pheR*$_{Re}$ promoters during growth on low phenol concentration (1.2 mM). Protocatechuate (3.5 mM) was used as an additional substrate in minimal medium BSM. *AU* arbitrary units. Standard deviations of three measurements are depicted by *error bars*

PheR activates the *pheA2* promoter

The *pheR* gene encodes an AraC-type transcriptional regulator and transcriptional patterns of *pheA2* and *pheR* suggested that PheR acts as an activator of the *pheA2A1* genes. The *pheA2* promoter region of *R. erythropolis* CCM2595 carries a typical tandem target sequence (González-Pérez et al. 1999) for AraC/XylS-type activators (Fig. 1), which we also found by genome searching in several *pheR–pheA2A1* clusters in various *Rhodococcus* strains (Fig. 3; see Discussion). However, all efforts to delete the *R. erythropolis* CCM2595 *pheR* gene using standard techniques of gene replacement failed (data not shown).

To examine whether the *R. erythropolis* CCM2595 PheR protein acts as an activator, we constructed the two-plasmid system in the heterologous host, *C. glutamicum*. *C. glutamicum* is a related bacterium in which promoters function similarly as in *R. erythropolis* (Knoppová et al. 2007) and its genome does not code for a PheR regulator. To test if the assumed binding site for PheR upstream of the P*pheA2*$_{Re}$ promoter (carried at the 100-bp DNA fragment; Fig. 3) is

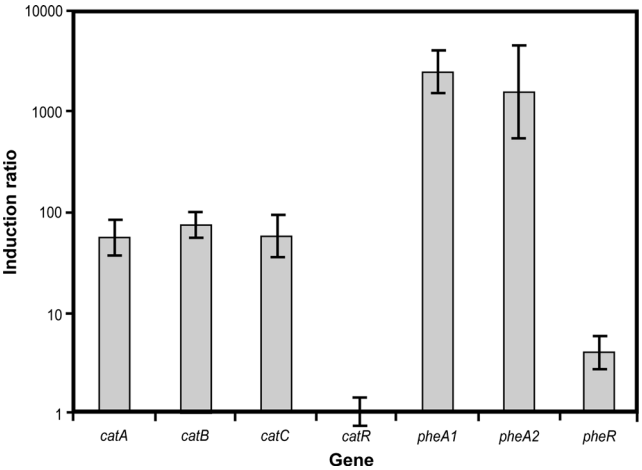

Fig. 6 Relative abundance of *cat* and *phe* transcripts in *R. erythropolis* CCM2595 cells grown on phenol and succinate determined by qPCR. The values obtained for the cultures grown on succinate were used as references and were set to 1.0 on the logarithmic scale. Averages of two independent biological replicates measured in triplicate are shown. Standard deviations are shown as *error bars*

involved in the promoter activation, we constructed a mutant promoter P*pheA2*M8 carrying 8 nt alteration within the supposed tandem PheR binding site upstream of the –35 region of P$pheA2_{Re}$ promoter (Fig. 3). Both pEC-XT99A*pheR* (expression vector with the *pheR* gene) and pEPRP*pheA2*WT (or pEPRP*pheA2*M8) were introduced into the *C. glutamicum* and the effect of *pheR* overexpression on P*pheA2* activity was measured by the reporter GFP fluorescence after IPTG addition. No significant promoter activity was observed for P$pheA2_{Re}$ or for P*pheA2*M8 in comparison with the control (empty pEPR1) in absence of *pheR* (Fig. 7). The P$pheA2_{Re}$activity increased markedly after the induction of *pheR* expression, whereas no increase in P*pheA2*M8 activity was observed under the same conditions. These results indicate that PheR is necessary for P*pheA2* function, and the altered nucleotides within the supposed PheR binding site are essential for promoter activation.

Discussion

The gene cluster *catABC–catR–pheR–pheA2A1* from *R. erythropolis* CCM2595 was found to encode four enzymes of the phenol degradation pathway and two regulatory proteins. Clusters with the same gene organization can be detected within the genome of *R. erythropolis* PR4 (NC_012490) and the genome shotgun sequences of *R. erythropolis* SK121 (ACNO01000001) and *R. qingshengii* BKS 20-40 (AODN01000022). The intergenic sequences *pheR–pheA2* in these strains are very similar to that of *R. erythropolis* CCM2595 (92 % to 100 % identity). We also found the same organization of the *cat–phe* genes within the sequenced parts of the genomes of the related strains, *R. pyridinivorans* AK37 (AHBW01000047), *R. ruber* 20-38 (AOEX01000015) and *Nocardia* sp. C-14 (DQ267826). The intergenic regions *pheR–pheA2* of these bacteria differ in terms of length and sequence distinctly from those of the first group. A different organization of the *phe* genes was found in *R. jostii* RHA1 and some other rhodococci. Two *pheR–pheA2A1* clusters (I and II), not adjacent to *catRABC*, were found in the genomes of *R. jostii* RHA1 (NC_008268) and *R. opacus* B4 (NC_012522). Similar *phe* I and *phe* II clusters can be found in *R. opacus* M213, *R. opacus* 1CP, *R. wratislaviensis* IFP 2016 and *R. imtechensis* RKJ300. No studies concerning regulation of *phe–cat* gene expression in the mentioned bacteria have been reported.

We proved that there is a single PHH encoded by *pheA2A1* in *R. erythropolis* CCM2595. Although a number of *Rhodococcus* strains were described as efficient phenol degraders useful in biotechnological processes (Paisio et al. 2012; Prieto et al. 2002), PHH, the key enzyme of phenol catabolism and expression of the *pheA2A1* genes were mostly not studied. Activity of PHH from *R. erythropolis* UVP1 that is closely similar to PHH from *R. erythropolis* CCM2595 was analyzed after expression of the respective *pheA2A1* genes into the two his-tagged PHH components in *E. coli* (Saa et al. 2009); however, expression control of these genes in *Rhodococcus* strains was not examined. We initiated the studies of regulatory mechanisms of the phenol degradation pathway in *Rhodococcus* by the analysis of expression of the *catABC* genes involved in the downstream part of the pathway (Veselý et al. 2007). We found that the expression of *catABC* is repressed by the IclR-type regulator CatR and induced by phenol in *R. erythropolis* CCM2595 (Veselý et al. 2007). Expression of the genes involved in the catabolism of aromatic compounds studied particularly in soil bacteria is controlled by a number of specific and global regulatory mechanisms which integrate diverse signals from the environment (Cases and de Lorenzo 2005). Most apparent regulatory phenomena are the induction of gene expression by the presence of the substrate and CCR of this induction exerted by more easily

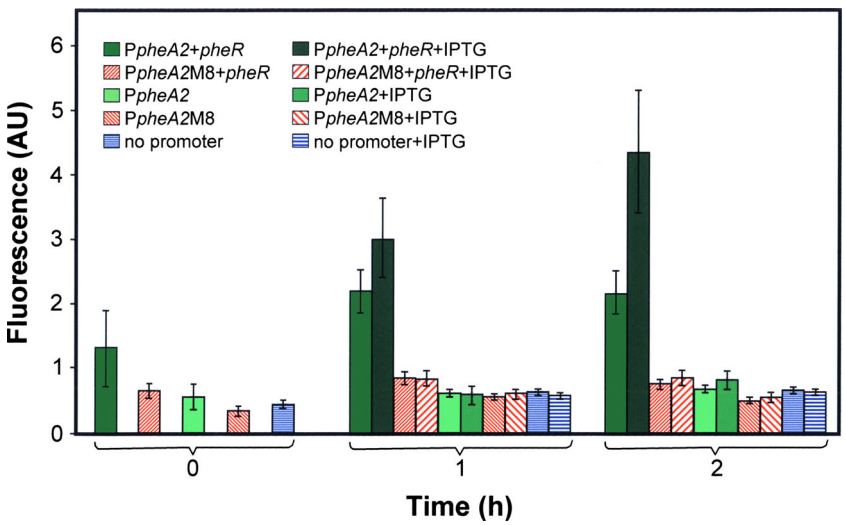

Fig. 7 Effect of PheR overexpression on the activity of P*pheA2* and P*pheA2*M8 promoters from *R. erythropolis* CCM2595. Promoter activity was determined by measuring the intensity of fluorescence of the GFP reporter. *AU*; arbitrary units. IPTG was added at time zero. Standard deviations of three measurements are depicted by *error bars*

metabolizable substrates (Rojo 2010). We proved that the induction of phenol hydroxylase is triggered at the level of transcription of the *pheR* and *pheA2A1* genes by the presence of phenol. The activity of the mPH was also found to be induced by phenol, e.g., in *Pseudomonas* strains (Shingler 2003) or in *Acinetobacter calcoaceticus* (Yu et al. 2011).However, no data on induction of the two-component PHH and modulation of expression of the respective genes mediated by a carbon catabolic repression or utilization hierarchy of aromatic substrates have been available until now.

Many soil bacteria carry sets of genes determining the catabolic activities which allow them to degrade and utilize a wide range of aromatic compounds, either of a natural origin (e.g., lignin metabolites) or xenobiotics (e.g., chlorophenols and polychlorinated biphenyls). Since mostly heterogenous mixtures of aromatic compounds of a natural or man-made origin are present in soil, a hierarchy of the substrate utilization evolved in most bacteria. Out of the aromatic compounds for which the substrate hierarchy was described (Nichols and Harwood 1995; Choi et al. 2007), we tested the effects of benzoate, *p*-hydroxybenzoate and protocatechuate on the phenol utilization. None of these aromatic substrates repressed the induction of PHH and utilization of phenol.

The presence of the simple, easily metabolizable carbon and energy sources, such as glucose or organic acids, in a mixture with aromatic compounds, generally results in the inhibition of the aromatic substrate catabolism by CCR. This phenomenon was observed in many bacterial species, but the mechanisms of CCR differ between bacterial species (Rojo 2010). We have already shown that glucose did not repress the catechol degradation pathway in *R. erythropolis* CCM2595 (Veselý et al. 2007). Similarly, expression of the *pheA2A1* genes was repressed neither by glucose nor glycerol (Fig. 4a). In contrast, we observed partial *pheA2A1* repression by succinate at the transcriptional level. The observed partial repression of the PHH activity by glucose (Table 3) which did not correlate with the results of the transcriptional analysis (Fig. 4a) may be mediated by an unknown posttranscriptional regulation. However, this partial PHH repression did not affect the flux through the phenol degradation pathway since phenol was degraded completely after 26 h independently of the presence of glucose (data not shown).

Different expression patterns were found in the two *R. jostii* RHA1 *phe* clusters. The transcription from the P*pheA2*$_{RjII}$ promoter was substantially repressed only by succinate (Fig. 4e), whereas P*pheA2*$_{RjI}$ activity was not decreased by any of the substrates tested in the presence of phenol (Fig. 4c). The diverse transcription patterns of the three *phe* clusters from *R. erythropolis* CCM2595 and *R. jostii* RHA1 during growth suggest that some subtle regulatory mechanisms of the gene expression differ in the individual cases. Different regulation of two *phe* genes in *R. jostii* suggests that the *phe* clusters I and II are involved in redundant catabolic reactions that may be triggered under different growth conditions. The temporal transcriptional patterns of P*pheA2* and P*pheR* were found to correlate in each of the P*pheA2*–P*pheR* pair (Fig. 4). Activity of P*pheA2*$_{Re}$ was delayed during the cultivation in comparison with P*pheR*$_{Re}$ when a low phenol concentration was used for growth (Fig. 5). We found that overexpression of *pheR* resulted in a sharp increase of P*pheA2*$_{Re}$ activity in the heterologous host, *C. glutamicum* (Fig. 7). A moderate increase of P*pheA2*$_{Re}$ activity was also apparent in the presence of pEC-XT99A*pheR* without addition of IPTG (due to the leaky *pheR* expression from the *trc* promoter). None of these effects was observed when *pheR* was missing or the assumed PheR binding site was altered. These results suggest that PheR is an activator of the *pheA2A1* genes. AraC-type regulator NphR was also found to activate transcription of the genes encoding two-component 4-nitrophenol hydroxylase in the *Rhodococcus* sp., strain PN1. However, only 4-nitrophenol induced nitrophenol degradation pathway and expression of the *nphR* gene was found to be constitutive, in the PN1 strain (Takeo et al. 2008). Inspection of the *pheA2* promoter sequence of all three *phe* analyzed clusters showed that the tandem binding sites for the AraC-type regulator are present at the exactly same position overlapping two nucleotides of the putative –35 promoter hexamer of P*pheA2* (Fig. 3). The proposed PheR$_{Re}$ binding site shares 15 nt out of 18 nt of the AraC/XylS-type activator binding site upstream of the Pm promoter which drives transcription of the genes involved in alkylbenzoates catabolic pathway in *P. putida* (González-Pérez et al. 1999). In both cases the downstream GGNT$^{A}/_{C}$ motif overlaps by 2 nt with the –35 hexamer.

To isolate the PheR protein from *R. erythropolis* CCM2595 and use it for direct biochemical and biophysical analyses, e.g., the electrophoretic mobility shift assay, which may directly prove binding of the protein to the supposed target site, we have overexpressed the *pheR* gene in *E. coli* and purified the PheR protein. However, the procedure yielded mostly insoluble inclusion bodies (data not shown), which is a typical feature of the AraC-family proteins (Schleif 2010). Alignment of the *pheA2–pheR* promoter regions from *R. erythropolis* and *R. jostii* with the respective parts of the sequences of the homologous *phe* clusters from the related strains showed the similarity of the key regulatory sequences in these bacteria (Fig. 3). Nearly identical potential binding sites for a CRP-type regulator were detected within the *pheR* promoter regions of all aligned sequences (Fig. 3). Closely similar sequence CTGTGT–N6–TCACAG within the promoters of two genes involved in tetralin utilization was proved to be involved in binding of the CRP-like protein which exerted glucose-mediated CCR in *Rhodococcus* sp. TFB strain (Tomás-Gallardo et al. 2012). Potential function of the CRP-like protein from *R. erythropolis* CCM2595 and *R. jostii* RHA1 in CCR or its connection to the *pheR* gene expression in *Rhodococcus* strains remain to be elucidated.

Acknowledgments This work was supported by a grant (2B08062 *AROMAGEN*) from the Czech Ministry of Education, Youth and Sports, grant P504/11/0394 from Czech Science Foundation and internal project RVO61388971 (Institute of Microbiology).

References

Bleichrodt FS, Fischer R, Gerischer UC (2010) The β-ketoadipate pathway of *Acinetobacter baylyi* undergoes carbon catabolite repression, cross-regulation and vertical regulation, and is affected by Crc. Microbiology 156:1313–1322

Bradford MA (1972) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of dye binding. Anal Biochem 72:248–252

Cases I, de Lorenzo V (2005) Promoters in the environment: transcriptional regulation in its natural context. Nat Rev Microbiol 3:105–118

Choi KY, Zylstra GJ, Kim E (2007) Benzoate catabolite repression of the phthalate degradation pathway in *Rhodococcus* sp. strain DK17. Appl Environ Microbiol 73:1370–1374

Duffner FM, Müller R (1998) A novel phenol hydroxylase and catechol 2,3-dioxygenase from the thermophilic *Bacillus thermoleovorans* strain A2: nucleotide sequence and analysis of the genes. FEMS Microbiol Lett 161:37–45

Dusch N, Pühler A, Kalinowski J (1999) Expression of the *Corynebacterium glutamicum panD* gene encoding L-aspartate-α-decarboxylase leads to pantothenate overproduction in *Escherichia coli*. Appl Environ Microbiol 65:1530–1539

González-Pérez MM, Ramos JL, Gallegos MT, Marqués S (1999) Critical nucleotides in the upstream region of the XylS-dependent TOL *meta*-cleavage pathway operon promoter as deduced from analysis of mutants. J Biol Chem 274:2286–2290

Hanahan E (1985) Techniques for transformation of *E. coli*. In: Glover DM (ed) DNA cloning. A practical approach, vol 1. IRL, Oxford, pp 109–135

Hernandez MA, Mohn WW, Martinez E, Rost E, Alvarez AF, Alvarez HM (2008) Biosynthesis of storage compounds by *Rhodococcus jostii* RHA1 and global identification of genes involved in their metabolism. BMC Genomics 9:600

Holátko J, Elišáková V, Prouza M, Sobotka M, Nešvera J, Pátek M (2009) Metabolic engineering of the L-valine biosynthesis pathway in *Corynebacterium glutamicum* using promoter activity modulation. J Biotechnol 139:203–210

Kim IC, Oriel PJ (1995) Characterization of the *Bacillus stearothermophilus* BR219 phenol hydroxylase gene. Appl Environ Microbiol 61:1252–1256

Kirchner O, Tauch A (2003) Tools for genetic engineering in the amino acid-producing bacterium *Corynebacterium glutamicum*. J Biotechnol 104:287–299

Kirchner U, Westphal AH, Müller R, van Berkel WJ (2003) Phenol hydroxylase from *Bacillus thermoglucosidasius* A7, a two-protein component monooxygenase with a dual role for FAD. J Biol Chem 278:47545–47553

Knoppová M, Phensaijai M, Veselý M, Zemanová M, Nešvera J, Pátek M (2007) Plasmid vectors for testing in vivo promoter activities in *Corynebacterium glutamicum* and *Rhodococcus erythropolis*. Curr Microbiol 55:234–239

Larkin MJ, Kulakov LA, Allen CC (2005) Biodegradation and *Rhodococcus*—masters of catabolic versatility. Curr Opin Biotechnol 16:282–290

Martin RW (1949) Rapid colorimetric estimation of phenol. Nature 21: 1419–1420

Martínková L, Uhnáková B, Pátek M, Nešvera J, Křen V (2009) Biodegradation potential of the genus *Rhodococcus*. Environ Int 35:162–177

McLeod MP, Warren RL, Hsiao WW, Araki N, Myhre M, Fernandes C, Miyazawa D, Wong W, Lillquist AL, Wang D, Dosanjh M, Hara H, Petrescu A, Morin RD, Yang G, Stott JM, Schein JE, Shin H, Smailus D, Siddiqui AS, Marra MA, Jones SJ, Holt R, Brinkman FS, Miyauchi K, Fukuda M, Davies JE, Mohn WW, Eltis LD (2006) The complete genome of *Rhodococcus* sp. RHA1 provides insights into a catabolic powerhouse. Proc Natl Acad Sci U S A 103:15582–15587

Müller C, Petruschka L, Cuypers H, Burchhardt G, Herrmann H (1996) Carbon catabolite repression of phenol degradation in *Pseudomonas putida* is mediated by the inhibition of the activator protein PhlR. J Bacteriol 178:2030–2036

Neujahr HY, Gaal A (1973) Phenol hydroxylase from yeast. Purification and properties of the enzyme from *Trichosporon cutaneum*. Eur J Biochem 35:386–400

Nichols NN, Harwood CS (1995) Repression of 4-hydroxybenzoate transport and degradation by benzoate: a new layer of regulatory control in the *Pseudomonas putida* β-ketoadipate pathway. J Bacteriol 177:7033–7040

Omokoko B, Jäntges UK, Zimmermann M, Reiss M, Hartmeier W (2008) Isolation of the *phe*-operon from *G. stearothermophilus* comprising the phenol degradative *meta*-pathway genes and a novel transcriptional regulator. BMC Microbiol 8:197

Paisio CE, Talano MA, Gonzalez PS, Busto VD, Talou JR, Agostini E (2012) Isolation and characterization of a *Rhodococcus* strain with phenol-degrading ability and its potential use for tannery effluent biotreatment. Environ Sci Pollut Res Int 19:3430–3439

Pátek M, Muth G, Wohlleben W (2003) Function of *Corynebacterium glutamicum* promoters in *Escherichia coli*, *Streptomyces lividans*, and *Bacillus subtilis*. J Biotechnol 104:325–334

Prieto MB, Hidalgo A, Rodriguez-Fernandez C, Serra JL, Llama MJ (2002) Biodegradation of phenol in synthetic and industrial wastewater by *Rhodococcus erythropolis* UPV-1 immobilized in an air-stirred reactor with clarifier. Appl Microbiol Biotechnol 58:853–859

Putrinš M, Tover A, Tegova R, Saks Ü, Kivisaar M (2007) Study of factors which negatively affect expression of the phenol degradation operon *pheBA* in *Pseudomonas putida*. Microbiology 153:1860–1871

Rojo F (2010) Carbon catabolite repression in *Pseudomonas*: optimizing metabolic versatility and interactions with the environment. FEMS Microbiol Rev 34:658–684

Saa L, Jaureguibeitia A, Largo E, Llama MJ, Serra JL (2009) Cloning, purification and characterization of two components of phenol hydroxylase from *Rhodococcus erythropolis* UPV-1. Appl Microbiol Biotechnol 86:201–211

Sambrook J, Russel DV (2001) Molecular cloning. A laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schleif R (2010) AraC protein, regulation of the L-arabinose operon in *Escherichia coli*, and the light switch mechanism of AraC action. FEMS Microbiol Rev 34:779–796

Shingler V (2003) Integrated regulation in response to aromatic compounds: from signal sensing to attractive behaviour. Environ Microbiol 5:1226–1241

Shingler V, Bartilson M, Moore T (1993) Cloning and nucleotide sequence of the gene encoding the positive regulator (DmpR) of the phenol catabolic pathway encoded by pVI150 and identification of DmpR as a member of the NtrC family of transcriptional activators. J Bacteriol 175:1596–1604

Takeo M, Murakami M, Niihara S, Yamamoto K, Nishimura M, Kato D, Negoro S (2008) Mechanism of 4-nitrophenol oxidation in *Rhodococcus* sp. Strain PN1: characterization of the two-component 4-nitrophenol hydroxylase and regulation of its expression. J Bacteriol 190:7367–7374

Teramoto M, Harayama S, Watanabe K (2001) PhcS represses gratuitous expression of phenol-metabolizing enzymes in *Comamonas testosteroni* R5. J Bacteriol 183:4227–4234

Tomás-Gallardo L, Santero E, Floriano B (2012) Involvement of a putative cyclic AMP receptor protein (CRP)-like binding sequence and a CRP-like protein in glucose-mediated catabolite repression of *thn* genes in *Rhodococcus* sp. strain TFB. Appl Environ Microbiol 78:5460–5462

van der Geize R, Dijkhuizen L (2004) Harnessing the catabolic diversity of rhodococci for environmental and biotechnological applications. Curr Opin Microbiol 7:255–261

Veselý M, Pátek M, Nešvera J, Čejková A, Masák J, Jirků V (2003) Host–vector system for phenol-degrading *Rhodococcus erythropolis* based on *Corynebacterium* plasmids. Appl Microbiol Biotechnol 61:523–527

Veselý M, Knoppová M, Nešvera J, Pátek M (2007) Analysis of *catRABC* operon for catechol degradation from phenol-degrading *Rhodococcus erythropolis*. Appl Microbiol Biotechnol 76:159–168

Yu H, Peng Z, Zhan Y, Wang J, Yan Y, Chen M, Lu W, Ping S, Zhang W, Zhao Z, Li S, Takeo M, Lin M (2011) Novel regulator MphX represses activation of phenol hydroxylase genes caused by a XylR/DmpR-type regulator MphR in *Acinetobacter calcoaceticus*. PLoS ONE 6:e17350

Zídková L, Szököl J, Rucká l, Pátek M, Nešvera J (2013) Biodegradation of phenol using recombinant plasmid-carrying *Rhodococcus erythropolis* strains. Int Biodeter Biodegradation 84:179–184