

Analysis of *catRABC* operon for catechol degradation from phenol-degrading *Rhodococcus erythropolis*

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Abstract The gene cluster *catRABC*, involved in catechol degradation, was isolated from *Rhodococcus erythropolis* CCM2595. The genes *catA*, *catB*, *catC*, and the divergently transcribed *catR* code for catechol 1,2-dioxygenase, *cis,cis*-muconate cycloisomerase, muconolactone isomerase, and an IclR-type transcriptional regulator, respectively. Measurements of catechol 1,2-dioxygenase activity showed that the expression of *catA* is induced by phenol but not by catechol or *cis,cis*-muconate. The activity of catechol 1,2-dioxygenase was repressed by succinate, but no repression by glucose was observed. The transcription start points of *catA* and *catR* were determined by primer extension analysis, and the respective promoters (P-*catA* and P-*catR*) were thus localized. Measurements of promoter activity during batch cultivation using transcriptional fusion with the *gfpuv* reporter gene showed that expression of the *catR-catABC* operon is regulated at the level of transcription. Both P-*catR* and P-*catA* are repressed by CatR, and the induction of P-*catA* by phenol is maintained in the absence of the repressor (in *R. erythropolis* Δ *catR*). Two different potential binding sites for the IclR-type regulator and a recognition site for the cyclic AMP receptor protein (CRP) were identified within the intergenic region between *catR* and *catA*.

Introduction

A number of aerobic biodegradation pathways of aromatic compounds like phenol, benzoate, or naphthalene converge into catechol ring cleavage. The scission of the aromatic ring is catalyzed by dioxygenases in bacteria and occurs through *ortho*- or *meta*-cleavage. *Ortho*-cleavage is followed by further steps of the β -keto adipate pathway, which finally converts the produced metabolites into intermediates of the citrate cycle. Other aromatic compounds, like *p*-cresol and *p*-hydroxybenzoate are converted to β -keto adipate via protocatechuate (Harwood and Parales 1996). In various Gram-negative bacteria (e.g., in *Pseudomonas putida* or *P. resinovorans*), the genes *catA*, *catB*, and *catC* (coding for catechol 1,2-dioxygenase, *cis,cis*-muconate cycloisomerase, and muconolactone isomerase, respectively) involved in the degradation of catechol to β -keto adipate form an operon transcribed divergently from the *catR* gene, coding for a LysR-type transcriptional regulator. In these bacteria, the CatR regulator acts as an activator of the adjacent catabolic genes by binding to the sites upstream of the *cat* operon in the presence of an inducer, which is usually a pathway intermediate like *cis,cis*-muconate (McFall et al. 1998; Nojiri et al. 2002). The gene coding for the LysR-type regulator was also found to be clustered to the genes *catA*, *catB*, and *catC* in the Gram-positive bacterium *Arthrobacter* sp. (Murakami et al. 2004). The regulatory *catR* gene transcribed divergently from the catabolic genes *catABC* was described in *Rhodococcus opacus* 1CP as well; however, the regulatory protein was a member of the IclR family of regulators (Eulberg and Schlömann 1998). IclR-type regulators are generally transcriptional repressors, although IclR proteins, which regulate the expression of catabolic genes, may also function as

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activators (Tropel and van der Meer 2004). The involvement of CatR in the regulation of *catABC* expression in *R. opacus* 1CP was proved by the binding of the protein within the intergenic *catR-catA* region (Eulberg and Schlömann 1998).

The Gram-positive actinobacterium *Rhodococcus erythropolis* CCM2595 was shown to utilize phenol, catechol, resorcinol, hydroxybenzoate, hydroquinone, and *p*-chlorophenol (Čejková et al. 2005). Its potential for biotechnological applications was studied due to the significance of the strain in bioremediation. Its cells were more resistant to toxic compounds in the form of a biofilm and in the presence of humic acids and were able to degrade higher concentrations of these substrates under these conditions (Čejková et al. 2005; Masak et al. 2005). We have developed a host-vector system for this strain (Veselý et al. 2003) as well as techniques for genetic manipulations within the chromosome (Čejková et al. 2005) and expressed the *gfp* gene coding for green fluorescent protein (GFP; Veselý et al. 2003). As catechol dioxygenases play a key role in the catabolism of various aromatic compounds, converting aromatics to aliphatics, we focused on the cloning and characterization of the genes involved in catechol degradation. In this paper, we describe the isolation of the *catR-catABC* operon from *R. erythropolis* CCM2595, induction of catechol 1,2-dioxygenase, and determination and analysis of the promoters driving transcription of the *catR* gene and *catABC* operon.

Materials and methods

Strains and vectors

The strain *Escherichia coli* DH5 α (Hanahan 1985) was used for cloning, *E. coli* S17-1 (Simon et al. 1983) as a donor for conjugation and the strain *R. erythropolis* CCM2595 (Veselý et al. 2003) was the source of *cat* genes. The plasmids used are shown in Table 1.

Growth conditions

E. coli was grown in Luria–Bertani (LB) medium at 37°C. *R. erythropolis* CCM2529 was grown in LBP medium (van der Geize et al. 2001) with 0.5% glucose and 1.5% Tween 80 (used against cell clumping) at 30°C or in basal salt minimal medium (BSM; Ogawa and Miyashita 1995) with various carbon sources (phenol, catechol, benzoate, *p*-hydroxybenzoate, protocatechuate, *cis,cis*-muconate; each in concentration 1.7 mmol/l, glucose; 55 mmol/l, succinate; 37 mmol/l). The selection media contained kanamycin (30 μ g/ml for *E. coli* and 200 μ g/ml for *R. erythropolis*).

DNA manipulations, cloning, and sequencing

DNA isolation, polymerase chain reaction (PCR), transformation of *E. coli*, DNA cloning, and DNA analysis were done by standard methods (Sambrook and Russel 2001). Plasmid DNA was isolated from *R. erythropolis* by the same method (alkaline lysis) as from *E. coli*, with the following modifications. Before lysis, *R. erythropolis* cells were incubated for 2 h with lysozyme (5 mg/ml). For the lysis, a higher concentration of sodium dodecyl sulfate (5%) was used. Genomic DNA from *R. erythropolis* was isolated as described previously (Treadway et al. 1999). The primers for PCR amplification of the internal *catA* fragment were CDOF1 (CAGGATCCAGGGCCCGT/CACTAC) and CDOR1 (TCGGATCCGACCCAGT CACC). Total DNA from *R. erythropolis* CCM2595 was used as a template. The fragment was cloned into the *Bam*HI site of pK18mobsacB, the resulting construct pCATA2 was introduced into the cells of *R. erythropolis* by conjugation, and after integration of the plasmid into the chromosome, regions flanking the cloned part of *catA* were isolated by the plasmid-rescue technique. The sequence of the complete *cat* cluster (3,650 bp) was determined by primer walking using the ABI Prism 2100 sequencer (Perkin Elmer). Deletion within the *catR* gene of *R. erythropolis* was constructed using the plasmid pDCATR

Table 1 Plasmids used in this study

Plasmid	Relevant characteristics	Reference
pK18mobsacB	<i>E. coli</i> vector for chromosomal integration, Km ^R	Schäfer et al. (1994)
pSRK21	<i>E. coli</i> – <i>R. erythropolis</i> vector, Km ^R	Veselý et al. (2003)
pCATA2	Internal fragment of <i>catA</i> in pK18mobsacB	This work
pSRK21catRABC	Complete <i>catRABC</i> cluster	This work
pDCATR	<i>E. coli</i> vector for <i>catR</i> deletion	This work
pEPR1	<i>E. coli</i> – <i>Rhodococcus</i> promoter-probe vector, promoter-less <i>gfpuv</i> as reporter	Knoppová et al., submitted
pEPRcatA	pEPR1 carrying the <i>catA</i> promoter	This work
pEPRcatR	pEPR1 carrying the <i>catR</i> promoter	This work

consisting of pK18mobsacB and a fragment of the *catRABC* cluster (1,247 bp) with a deletion of 103 bp within *catR*. The plasmid was introduced into *R. erythropolis* by conjugation and clones in which the double recombination event occurred were identified by positive selection based on the conditional lethal effect of the *sacB* gene in *R. erythropolis* (Schäfer et al. 1994). The clones, free of the vector sequences and carrying the *catR* deletion, were identified by PCR and sequencing. Using the same gene replacement technique and an analogous procedure, the *R. erythropolis* Δ *catA* clone with the 175-bp internal deletion was constructed.

RNA isolation and primer extension analysis

RNA from *R. erythropolis* cultures was isolated by phenol extraction as described by Eikmanns et al. (1994). Reverse transcription (RT) was performed with SuperScript II transcriptase (Invitrogen) and fluorescein-labeled primer GFP1 (CTAATTCACAAGAATTGGGAC) complementary to the vector pEPR1, as described previously (Pátek et al. 2003). The reverse transcript was run in an automatic ALF DNA Sequencer (Pharmacia Biotech) with the DNA sequencing reactions generated by using the same labeled primer.

RT-PCR analysis

The total RNA isolated from the cells of *R. erythropolis* cultivated on phenol was incubated with DNase (free of RNase; TopBio, Czech Republic) to remove traces of contaminating DNA. The template RNA (0.6 μ g) was then transcribed into complementary DNA (cDNA) using SuperScript II reverse transcriptase and primer CATCR1 [CAGGGGCAGGTTCCAGAGA, nucleotides (nt) at positions 3,345 to 3,327 of the *catRABC* sequence, reverse]. Reverse transcription product (4 μ l) was used in subsequent PCR reactions (in 50 μ l) using Taq polymerase (TopBio). The primers CATA8 (CGAGAACGACGTGGCAACT, nt 1,851 to 1,869, forward) and CATBR1 (GATGTACTTCTCGATGATGACC, nt 2,213 to 2,192, reverse) were used to amplify the fragment AB (361 bp), whereas primers CATB3 (ATCGCCCTCAAGACCAC, nt 2,796 to 2,812, forward) and CATCR1 were used for the fragment BC (549 bp). All enzyme reactions were performed following the protocols recommended by the manufacturers. Products were analyzed by electrophoresis in a 1.2% agarose gel.

Transformation and conjugation of *R. erythropolis*

R. erythropolis cells were transformed by electroporation as described previously (Vesely et al. 2003). Conjugation was done essentially according to van der Geize et al. (2001),

using the *E. coli* S17-1 (Simon et al. 1983) as a donor. The cells of *R. erythropolis* and *E. coli* were mixed and cultivated on LBP plates at 30°C for 24 h. Transconjugants were selected on LBP plates containing nalidixic acid (50 μ g/ml) and kanamycin (200 μ g/ml).

Enzyme assay

R. erythropolis cells were disrupted with an ultrasonic disintegrator (Ikasonic U50, IKA Labortechnik, Staufen, Germany), and after centrifugation (30 min; 18,000 \times g; 4°C), the activity of catechol 1,2-dioxygenase was measured spectrophotometrically in a cell-free extract using the method of Ngai et al. (1990). Specific activity was expressed as micromoles of *cis,cis*-muconate per minute per milligram of protein (U/mg protein).

GFP fluorescence intensity measurement

The cell suspension was washed twice with phosphate-buffered saline (PBS) (8.25 g Na₂HPO₄, 2.05 g NaH₂PO₄, 4 g NaCl, in 1,000 ml, pH 7.4), diluted with PBS to OD₆₀₀=0.2 and its fluorescence measured with a Shimadzu RF-540 spectrofluorometer (excitation wavelength, 395 nm; emission wavelength, 465–600 nm; maximum at 509 nm). Fluorescence intensity was expressed in arbitrary units (AU) normalized to cell density.

Nucleotide sequence accession number

The nucleotide sequence of the *catRABC* gene cluster was deposited in GenBank under accession no. AJ605581.

Results

Cloning and sequencing of the internal fragment of *catA*

The genes coding for catechol-degrading enzymes were searched for in *R. erythropolis* CCM2595. As no activity of catechol 2,3-dioxygenase was detected in this strain using a simple biochemical test (Worsey et al. 1978), we designed primers for cloning the *catA* gene for catechol 1,2-dioxygenase (C1,2DO). The primers were based on the conserved regions of C1,2DO within the sequences from GenBank: *Arthrobacter* sp. (M94318), *P. putida* (AY029000), *R. opacus* (X99622), and *Streptomyces setonii* (AF277051). The amplified PCR fragment (genomic DNA of *R. erythropolis* CCM2595 was used as a template) of the appropriate size (about 400 bp) was cloned in the vector pK18mobsacB and sequenced. The nucleotide sequence revealed 68% identity with an internal section of *catA* from *R. opacus* 1CP according to database similarity

searches. We concluded that part of the *catA* gene from *R. erythropolis* CCM2595 had been cloned.

Isolation of the complete *catRABC* gene cluster and its sequencing

The regions flanking the cloned part of *catA* were isolated by the plasmid-rescue technique as follows: The plasmid with the *catA* fragment (designated pCATA2) was introduced into the cells of *R. erythropolis* by conjugation, and Km^R clones with the plasmid integrated (by homologous recombination) into the chromosome were identified using PCR. Total DNA of the resulting clone of *R. erythropolis*: pCATA2 was isolated, digested by *EcoRI* or *PstI*, and religated. After the transformation of *E. coli*, clones carrying the fragments flanking the original fragment of *catA* were isolated. Sequencing of the flanking fragments revealed the presence of four open reading frames. According to sequence similarity searches, these open reading frames correspond to the genes *catA*, *catB*, *catC*, and the divergently oriented *catR* (Fig. 1d) coding for catechol 1,2-dioxygenase, *cis,cis*-muconate cycloisomerase, muconolactone isomerase, and an IclR-type transcriptional regulator, respectively. The deduced amino acid sequences of the *catA*, *catB*, *catC*, and *catR* gene products from *R. erythropolis* CCM2595 showed the following degrees of identity with those from other strains of rhodococci: *Rhodococcus* sp. RHA1 (GenBank acc. no. CP000431; 73, 84, 81, and 72%, respectively), *R. opacus* 1CP (X99622; 72, 84, 80, and 71%, respectively), *Rhodococcus* sp. AN-22 (AB167712; 71, 72, and 87%, respectively; only the remnant of *catR* is present), *R. erythropolis* AN-13 (D83237; 71, 73, X, and 57%, respectively; X—the *catC* sequence is incomplete).

RT-PCR of the *catABC* gene cluster

To prove that the *catA* gene is cotranscribed with the genes *catB* and *catC*, a PCR involving an RT step was carried out with RNA isolated from phenol-grown *R. erythropolis* cells. The DNA fragments with expected sizes were amplified with the primers CATA8+CATBR1 spanning parts of the genes *catA* and *catB* and with the primers CATB3+CATCR1 spanning parts of the genes *catB* and *catC* when the same RT reaction product was used (data not shown). No PCR products were obtained when the RT step was omitted. These results indicate that a single transcript covers all three genes, which thus form an operon.

The *catA* gene codes for catechol 1,2-dioxygenase

To confirm the function of *catA*, an internal fragment (175 bp) of the chromosomal gene was deleted using the

gene replacement technique. The resulting strain *R. erythropolis* Δ *catA* grew in a minimal medium with glucose but did not grow in the medium with phenol (or catechol) as the only source of carbon. This result also showed that C1,2DO coded by *catA* codes for the only catechol-catabolizing enzyme present in phenol-degrading cells of in *R. erythropolis* CCM2595. Still another catechol dioxygenase might be present in the strain, which is induced with another substrate. C1,2DO activity was measured in cell extracts of *R. erythropolis* WT and *R. erythropolis* Δ *catA* cultivated with both glucose and phenol present in the medium. The specific C1,2DO activity was 0.25 and \leq 0.001 U/mg protein, respectively. The function of *catA* was confirmed by the observation that the specific C1,2DO activity in *R. erythropolis* carrying the *catRABC* cluster on the multicopy plasmid (pSRKcatRABC) was significantly higher than in the WT strain (0.71 U/mg protein). The plasmid pSRKcatRABC was also transferred to *E. coli*, and C1,2DO activity (0.2 U/mg protein) was found in transformants while no activity was detected in *E. coli* (pSRK21).

Activity of catechol 1,2-dioxygenase in *R. erythropolis* cells grown on various substrates

The specific activity of catechol 1,2-dioxygenase was measured in cell extracts of *R. erythropolis* CCM2595 grown in minimal medium with glucose, succinate, and phenol, both separately and in combination (Table 2). C1,2DO activity was apparently induced with phenol, whereas a very low activity was detected in extracts of cells grown with succinate or glucose. The strain grew poorly on catechol and *cis,cis*-muconate, and a very low activity of C1,2DO was also determined in the cell extracts from such cultures. When phenol and glucose were combined as substrates, C1,2DO activity was essentially the same as with phenol alone. Thus, no carbon catabolite repression was exerted by glucose. In contrast, when succinate was added to the medium with phenol, C1,2DO activity was only slightly above the basal level without induction (Table 2). This suggested that repression by succinate is involved in the control of *catA* expression.

Analysis of the promoters P-*catA* and P-*catR*

To analyze the transcriptional regulation of the *catR-catABC* gene cluster, the intergenic (*catR-catA*) DNA fragment (249 bp) also covering the 5'-ends (18 bp and 32 bp, respectively) of the divergently oriented coding regions was cloned in the promoter-probe vector pEPR1 in both orientations. The resulting constructs, pEPRPcatA and pEPRPcatR, were transferred to *R. erythropolis* by transformation. The colonies carrying pEPRPcatR exhibited

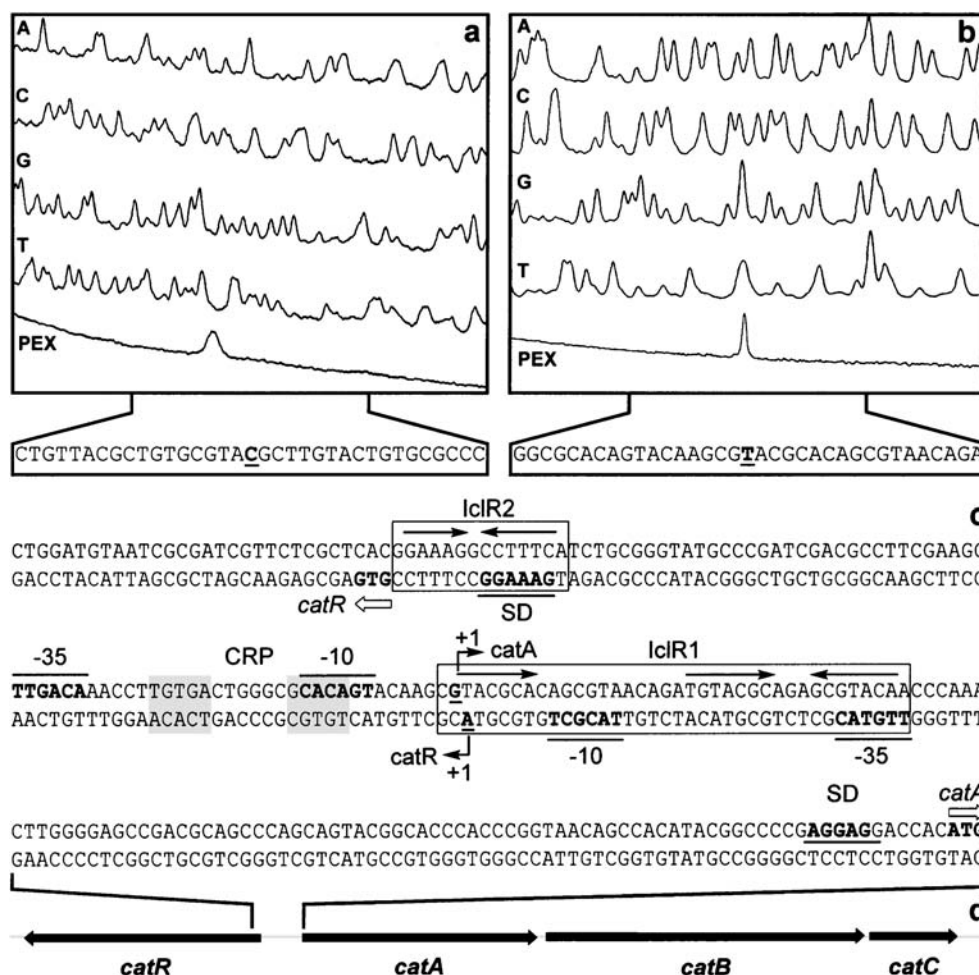


Fig. 1 Analysis of the *catR-catA* intergenic region. **a** Determination of the *catA* transcription start point (TSP). The bottom peak (PEX) represents cDNA synthesized in reverse transcription (primer extension) using RNA from *R. erythropolis*. The peaks generated by the automatic sequencer represent the products of sequencing reactions (A, C, G, T) performed with the same fluorescein-labeled primer as that used for primer extension. A portion of the nucleotide sequence derived from the sequencing signals is shown below. Note that the sequence is complementary to that shown in Fig. 1c. TSP (underlined) was determined by a comparison of the position of the primer

extension product and sequencing signal. **b** Determination of *catR* TSP. **c** Nucleotide sequence of the *catR-catA* intergenic region, regulatory features, and sequence elements. TSPs (+1) are underlined and transcription initiation indicated by the bent arrows. The proposed -35 and -10 hexamers and Shine-Dalgarno (SD) motifs are in bold letters. Database-deduced binding sites for the IclR-type regulator are boxed, and inverted and direct repeats are denoted by arrows within the boxes. The consensus-matching CRP-binding site is shaded. Translation initiation sites (in bold) are indicated by hollow arrows. **d** Scheme of the *catRABC* gene cluster

Table 2 Activity of catechol 1,2-dioxygenase in extracts of *R. erythropolis* cells grown in various substrates and their combinations

Substrate	Specific activity ^a (U/mg protein)
Glucose	0.003
Succinate	0.002
Phenol	0.253
Catechol	0.002
<i>cis,cis</i> -muconate	0.001
Phenol+glucose	0.295
Phenol+succinate	0.011

^aAverages of at least four independent measurements are shown. Standard deviation did not exceed 22% in any data set.

clear green fluorescence on the plates with minimal or complete medium with or without phenol, whereas the colonies with pEPRP*catA* exhibited fluorescence only in the presence of phenol in BSM medium. To localize the promoters of *catA* and *catR*, the transcription start points (TSPs) were determined by primer extension analyses (Fig. 1a,b) using RNA isolated from *R. erythropolis* (with the respective pEPR1 constructs) grown on BSM with phenol. The TSPs were found 117 and 84 nt upstream of the supposed translational starts of *catA* and *catR*, respectively (+1 in Fig. 1c). The -35- and -10-like elements of the promoter regions with appropriate spacing (TTGACA-17 nt-CACAGT-6 nt-TSP for *catA* and TTGTAC-17 nt-

TACGCT-6 nt-TSP for *catR*) were found within the sequences upstream of the TSPs. A clear signal indicating the same TSP of *catA* was also determined with the RNA isolated from *E. coli* (pEPRPcatA) grown in LB medium (not shown), whereas no signal for the *catR* gene was detected. This confirmed that *catA* may be expressed from its own promoter in *E. coli*.

The activities of the promoters P-*catA* and P-*catR* (transcriptionally fused to *gfpuv* in pEPR1) in the cells of *R. erythropolis* WT cultivated on various carbon substrates were examined using the reporter green fluorescence emitted by GFP. The activity of the *catA* promoter in glucose or succinate-grown cells of the *R. erythropolis* WT was low over the whole cultivation period and showed little variation (Fig. 2a). As expected, the activity of P-*catA* markedly increased with cultivation on phenol (Fig 2a) but not on catechol or *cis,cis*-muconate (data not shown). Activity of P-*catA* was also very weakly induced by benzoate (less than 10% as compared to phenol). Proto-

catechuate and *p*-hydroxybenzoate, aromatic substrates degraded by the protocatechuate branch of β -ketoacid pathway (Harwood and Parales 1996), did not induce the P-*catA* activity (data not shown). High activity of P-*catA* was also observed when the culture grew on a combination of phenol and glucose, whereas phenol in combination with succinate had almost no effect on P-*catA* activity in comparison with the growth on succinate alone. The results of these promoter studies were completely in agreement with the results of C1,2DO activity assays and demonstrated that both the induction of the enzyme activity by phenol and repression by succinate are controlled at the transcriptional level. Carbon catabolite repression is thus mediated by succinate but not by glucose. Repression of P-*catA*, similar to that caused by succinate, was also observed when the cells grew on a combination of phenol and protocatechuate or phenol and *p*-hydroxybenzoate. These substrates thus apparently also abolished the ability of phenol to induce expression of the operon.

The same analysis of the *catR* promoter function showed that P-*catR* displayed only low basal activity (0.1 to 0.4 AU as measured by GFP fluorescence) independently of the carbon source over the whole cultivation period.

To elucidate the role of CatR in the expression of the *catR* gene and the *catABC* operon, the *catR* gene was inactivated by the internal deletion of 103 bp. The activity of P-*catR* and P-*catA* was then measured again using transcriptional fusion with *gfpuv* (in pEPR1) in the resulting strain *R. erythropolis* Δ *catR*. The activity of the *catR* promoter in the Δ *catR* strain was about 10 to 100-fold higher (1.5 to 10.3 AU) than the basal activity in the WT strain in all media tested. In the culture with succinate (the best carbon source of those tested), P-*catR* activity was (in comparison with the WT-strain) 60-fold higher after 12 h and even 100-fold higher after 24 h of growth. These results demonstrated that the expression of the *catR* gene is regulated by a strong autorepression.

The activity of the *catA* promoter in the absence of functional CatR was about three- to fourfold higher on glucose, succinate, and succinate+phenol than in the WT (*catR*+) strain (Fig. 2). The observed increase in the basal P-*catA* activity in the Δ *catR* strain showed that CatR also acts as a repressor of P-*catA*. As in the WT strain, *catA* promoter activity in the Δ *catR* strain was significantly elevated during growth on phenol alone or on a combination of phenol with glucose.

Inspection of the *catR-catA* intergenic DNA sequence (199 bp) and DNA similarity searches in the GenBank database indicated that two different motifs with a high degree of identity with various binding sites for the regulators of the IclR family from different bacteria (Fig. 3a,c) are located within this region. In addition, an inverted repeat closely similar to the CRP (cyclic AMP

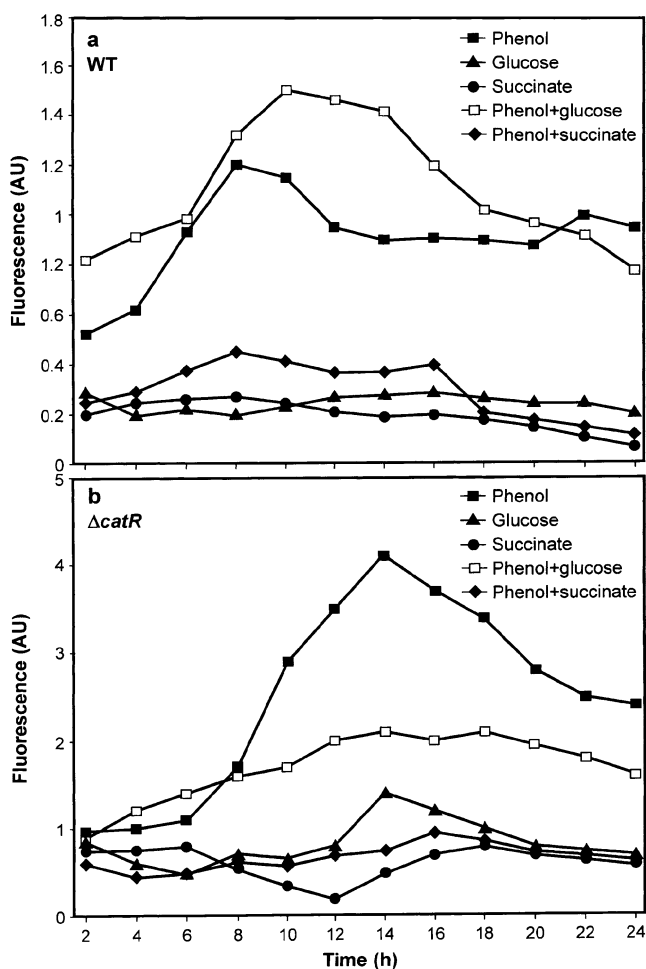


Fig. 2 Activity of the P-*catA* promoter on various substrates. Activity was determined as intensity of fluorescence of the GFP reporter. **a** *R. erythropolis* WT, **b** *R. erythropolis* Δ *catR*

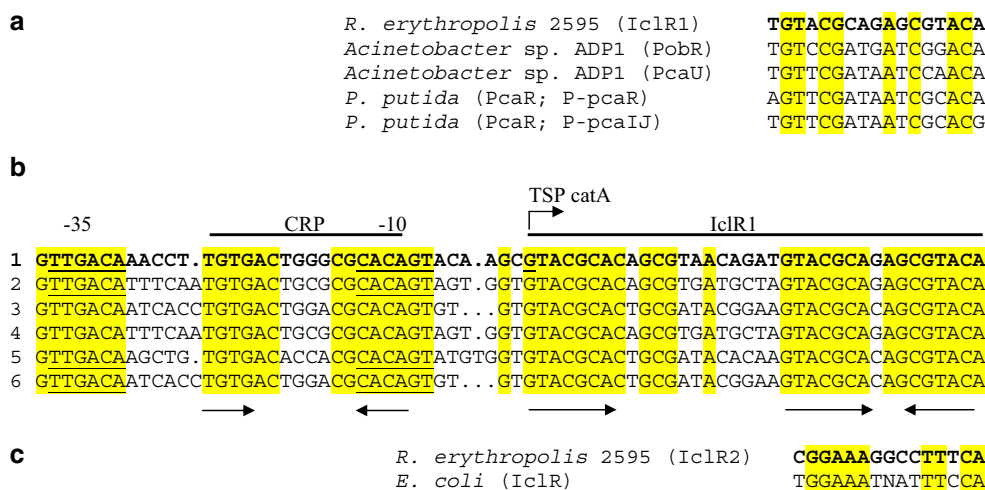


Fig. 3 Alignments of potential regulatory sequences from the intergenic region between *catR* and *catA* of *R. erythropolis* CCM2595. Nucleotides identical in all aligned sequences are shaded. *R. erythropolis* CCM2595 sequences are shown in bold. **a** IclR-box motif from *R. erythropolis* CCM2595 and experimentally proved IclR operator sequences from *Acinetobacter* sp. (PobR, PcaU; Gerischer et al. 1998) and *P. putida* (PcaR; *pcaR*, *pcaIJ* promoters; Guo and Houghton 1999). **b** Potential regulatory sequences from *R. erythropolis* CCM2595 (IclR1 and CRP) and sequences from *catR*–*catA*

region from *Rhodococcus* and *Nocardia* strains. (1) *R. erythropolis* CCM2595, (2) *R. opacus* 1CP (GenBank acc. no X99622), (3) *R. rhodochrous* NCIMB 13259 (AF043741), (4) *Rhodococcus* sp. RHA1 (CP000431), (5) *Rhodococcus* sp. AN-22 (AB167712), (6) *Nocardia* sp. C-14-1 (DQ267826). Inverted or direct repeats are indicated with arrows, the proposed –35 and –10 promoter elements are underlined, transcriptional start point (TSP) is denoted by a bent arrow and underlined. **c** Potential IclR2 box from *R. erythropolis* CCM2595 and consensus sequence of IclR binding sites from *E. coli* (Pan et al. 1996)

receptor protein) binding site from *E. coli* (Zheng et al. 2004) was detected within the sequence (Fig. 1c). As the IclR1 region and CRP motif overlap or are located immediately adjacent to the *catA* or *catR* promoters, we suppose that these potential binding sites are involved in the regulation of the expression of the *catR*–*catABC* operon.

Discussion

The cloned gene cluster *catR*–*catABC* of *R. erythropolis* CCM2595 forms a regulatory unit (an operon, as proved by RT-PCR analysis) responsible for the degradation of catechol to β -keto adipate enol-lactone that is further degraded via the β -keto adipate pathway to intermediates of the citrate cycle. The organization of the *catRABC* genes from *R. erythropolis* CCM2595 is closely similar to that of homologous genes in *R. opacus* 1CP (Eulberg and Schlömann 1998), *R. erythropolis* AN-13 (GenBank acc. no. D83237), *Rhodococcus* sp. RHA1 (CP000431), and *Nocardia* sp. C-14-1 (DQ267826). In all these cases, the CatR proteins coded by the genes transcribed divergently from the adjacent *catABC* operons are members of the IclR family of transcriptional regulators. Genes responsible for catechol degradation in *P. putida* (McFall et al. 1998) and *P. resinovorans* (Nojiri et al. 2002) are organized in the *catBCA* operons with *catR* genes also located upstream of *catB* in the opposite orientation. The operons *catRBAC*

and *catRBCA*-II, in which all genes are transcribed in the same direction, were discovered in thermophilic *S. setonii* (An et al. 2001) and in benzamide-assimilating *Arthrobacter* sp., respectively (Murakami et al. 2004). In all these and most other cases, expression of these catabolic genes is controlled by LysR-type transcriptional regulators. These regulators involved in the degradation of aromatic compounds function as transcriptional activators (Tropel and van der Meer 2004). The involvement of an IclR-type CatR regulator in the regulation of catechol-degradation genes, which was first found in *R. opacus* 1CP (Eulberg and Schlömann 1998), seems to be less frequent. IclR-type regulators function prevalently in the gene clusters that code for the degradation pathways of *p*-hydroxybenzoate and protocatechuate. Such genes (*pob* and *pca*) were found in *Acinetobacter baylyi* (DiMarco and Ornston 1994; Gerischer et al. 1998), in *P. putida* (Harwood and Parales 1996), in *R. opacus* (Eulberg et al. 1998), and in *Corynebacterium glutamicum* (Brinkrolf et al. 2006). Although the divergent arrangement of the regulatory genes (*pobR*, *pcaU*, *pcaR*, and *catR*) and degradative genes is similar to that found in operons under the control of LysR-type regulators, the position of the binding sites and their recognition sequences are different (Molina-Henares et al. 2006). In genes controlled by LysR-type regulators, intermediates like *cis,cis*-muconate or β -keto adipate are usually the inducers, whereas gene clusters under the regulation of IclR-type proteins are mostly induced by aromatic substrates like protocatechuate

(Gerischer et al. 1998), *p*-hydroxybenzoate (DiMarco and Ornston 1994), and gentisate (Brinkrolf et al. 2006). We have demonstrated that the activity of C1,2DO and the *catA* promoter are induced with phenol but not with *cis*, *cis*-muconate in *R. erythropolis* CCM2595. The activity of P-*catR* increased as much as 100-fold in the absence of functional CatR, which indicated that expression of the *catR* gene is autorepressed. This repression was independent of all the carbon sources tested and was not influenced by the inducer (phenol). No sensitivity of the autorepression to the inducers *p*-hydroxybenzoate and protocatechuate was observed in the *A. baylyi* ADP1 regulatory genes *pobR* (DiMarco and Ornston 1994) or *pcaU* (Gerischer et al. 1998). IclR-type proteins are generally repressors; however, some of the IclR-regulators that control degradative pathways have been shown to act as activators (Molina-Henares et al. 2006). According to our results from the comparison of P-*catA* activity in *R. erythropolis* Δ *catR* and the WT strain, the P-*catA* promoter is also repressed by CatR. In *C. glutamicum*, deletion of the *pcaR* regulator gene also resulted in increased levels of transcription of *pcaIJ* genes (Brinkrolf et al. 2006). In *A. baylyi* ADP1, the PcaU regulator only repressed the target genes (*pca* operon for protocatechuate utilization) in the absence of the inducer (protocatechuate), whereas it functioned as an activator in its presence. The inactivation of *pcaU* reduced the induced expression of *pca* structural genes by about 90% (Gerischer et al. 1998). PcaU thus mainly acts as an activator. In contrast, we have found that the activity of P-*catA* in the presence of the inducer (phenol) increased three- to fourfold with the deletion of *catR*. However, the activity of P-*catA* increased approximately eightfold in the presence of phenol at its maximum (Fig. 2) in comparison with the basal activity (on succinate) in both the Δ *catR* and *catR*⁺ strains. These results suggest that the main function of CatR is repression and that another mechanism of P-*catA* activation, which is not dependent on CatR, operates in *R. erythropolis* CCM2595. The genes *catABC*, similar in nucleotide sequence to *catABC* from *R. erythropolis* CCM2595, are constitutively expressed in *Rhodococcus* sp. AN-22 (Matsumura et al. 2006) during growth on both aromatic and non-aromatic substrates. The adjacent *catR* gene is disrupted by the IS204-like element in this strain, and the promoter within the IS element, which is therefore not regulated by original CatR, drives transcription of the operon. In *R. opacus*, NpdR, the IclR-type regulator, was found to repress the trinitrophenol degradation genes (Nga et al. 2004). In this case, deletion of the *npdR* gene resulted in an increase in hydrid transferase activity regardless of the presence of an inducer.

Transcriptional starts, promoter elements, and regulator binding sites (operators) were localized in the regions between the divergently oriented regulatory and target

genes *pobR-pobA* (134 bp) and *pcaU-pcaI* (286 bp) from *A. baylyi* ADP1. In these cases, the IclR binding sites (IclR boxes) consist of three short repeats, two forming a palindrome, and a direct repeat separated by 10 nt. Although the relative positions of the promoters and IclR boxes in *pobR-pobA* and *pcaU-pcaI* intergenic regions are different, the repeats are very similar (18 out of 21 nt identical). Nearly identical operators for the regulator PcaR were also found to control the expression of the genes *pcaI* and *pcaR* in *P. putida* (Guo and Houghton 1999). The apparent sequence similarity of the inverted repeat found within the *catR-catA* intergenic region (199 bp) of *R. erythropolis* CCM2595 (IclR1 in Fig. 1) to the repeats forming operators of the genes responsible for the degradation of *p*-hydroxybenzoate and protocatechuate suggests that it also serves as an operator for the control of *catR/catA* expression (Fig. 3a). Moreover, we have identified several conserved sequences of *catR-catA* intergenic regions from various strains of *Rhodococcus* and *Nocardia* in the GenBank database. These sequences share completely identical inverted and direct repeats forming a typical IclR binding site and the proposed –35 and –10 hexamers of P-*catA* (Fig. 3b). The regulators PobR and PcaU from *A. baylyi* (DiMarco and Ornston 1994; Gerischer et al. 1998) and PcaR from *P. putida* (Guo and Houghton 1999) act as activators. The operators for these proteins are located upstream of the promoters of the target genes. In contrast, CatR of *R. erythropolis* CCM2595 was shown to act as a repressor, and its potential operator overlaps the *catA* promoter (and also the *catR* promoter, Fig. 1c). This is in agreement with the theory that the regulatory function of transcriptional regulators is determined by the position of the binding site for the regulator relative to the TSP and not merely by the family of the protein (Madan Babu and Teichmann 2003). In *C. glutamicum*, the PcaR regulator, whose operator also overlaps the potential promoter of the target gene *pcaI*, was also shown to serve as a repressor (Brinkrolf et al. 2006).

Another 12-bp palindromic sequence, highly similar to the consensus of the IclR binding site in *E. coli* (Pan et al. 1996) (Fig. 3c), was found upstream of the translation start site of *catR* in *R. erythropolis* CCM2565. In this case, no counterparts were recognized in the homologous sequences of rhodococci and nocardiae.

We found that induction of the *catA* promoter and C1,2DO activity with phenol was suppressed by succinate in the growth medium, whereas the presence of glucose did not influence it. This suggests that a kind of carbon catabolite repression affects the transcription of *catA*. In a number of cases, the preferred substrates prevent the induction of genes involved in the degradation of aromatic compounds (Collier et al. 1996; McFall et al. 1998). The repression of enzymes involved in the degradation of *p*-

hydroxybenzoate and protocatechuate with succinate but not with glucose was described in *A. baylyi* ADP1 (Dal et al. 2002). It is worth mentioning that a conserved sequence, which only differs from the *E. coli* CRP consensus binding site TGTGA-N6-TCACA (Zheng et al. 2004) by a single nt, overlaps the *catA* promoter of *R. erythropolis* CCM2595 as well as the *catA* promoters within various *Rhodococcus* and *Nocardia catR-catA* sequences aligned in Fig. 3b. The gene coding for the regulatory protein of the CRP family (RHA1_ro04321) was found in the genome of *Rhodococcus* sp. RHA1 (the only *Rhodococcus* genome sequence available in the GenBank database).

R. erythropolis CCM 2595 grows well on *p*-hydroxybenzoate and protocatechuate, substrates degraded via the protocatechuate branch of β -keto adipate pathway. The pathways for catechol and protocatechuate degradation represent metabolically parallel but distinct branches of degradation of aromatic compounds (Harwood and Parales 1996). Bacteria usually show some preference (hierarchy) in aromatic carbon source utilization when cultivated in two or more substrates. *A. baylyi* ADP1 was shown to consume benzoate before *p*-hydroxybenzoate in the presence of both carbon sources (Gaines et al. 1996). Repression of the *catA* promoter by protocatechuate and *p*-hydroxybenzoate in *R. erythropolis* CCM2595 suggests that these substrates are utilized preferentially to phenol.

The presence of two different IclR boxes and a potential CRP binding site within the *catR-catA* intergenic region seems unique among the regulatory regions of genes controlled by IclR transcriptional regulators. Adjacent to the *catR* gene, we have identified the genes coding for small and large subunits of phenol hydroxylase (*pheA2*, *pheA1*) and for an AraC-type transcriptional regulator (*pheR*). Future work will focus on the analysis of the *R. erythropolis* CCM2595 *pheRA2A1* operon (GenBank AJ973228) and a study of the possible mechanisms of cross-regulation with *catRABC* genes.

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