Antonie van Leeuwenhoek (2014) 105:1179–1190
DOI 10.1007/s10482-014-0179-3

ORIGINAL PAPER

Expression control of nitrile hydratase and amidase genes in *Rhodococcus erythropolis* and substrate specificities of the enzymes

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Received: 27 November 2013 / Accepted: 16 April 2014 / Published online: 30 April 2014

Abstract Bacterial amidases and nitrile hydratases can be used for the synthesis of various intermediates and products in the chemical and pharmaceutical industries and for the bioremediation of toxic pollutants. The aim of this study was to analyze the expression of the amidase and nitrile hydratase genes of *Rhodococcus erythropolis* and test the stereospecific nitrile hydratase and amidase activities on chiral cyanohydrins. The nucleotide sequences of the gene clusters containing the *oxd* (aldoxime dehydratase), *ami* (amidase), *nha1*, *nha2* (subunits of the nitrile hydratase), *nhr1, nhr2, nhr3* and *nhr4* (putative regulatory proteins) genes of two *R. erythropolis* strains, A4 and CCM2595, were determined. All genes of both of the clusters are transcribed in the same direction. RT-PCR analysis, primer extension and promoter fusions with the *gfp* reporter gene showed that the *ami*, *nha1* and *nha2* genes of *R. erythropolis* A4 form an operon transcribed from the P*ami* promoter and an internal P*nha* promoter. The activity of P*ami* was found to be weakly induced when the cells grew in the presence of acetonitrile, whereas the P*nha* promoter was moderately induced by both the acetonitrile or acetamide used instead of the inorganic nitrogen source. However, *R. erythropolis* A4 cells showed no increase in amidase and nitrile hydratase activities in the presence of acetamide or acetonitrile in the medium. *R. erythropolis* A4 nitrile hydratase and amidase were found to be effective at hydrolysing cyanohydrins and 2-hydroxyamides, respectively.

Keywords *Rhodococcus erythropolis* · Amidase · Nitrile hydratase · Gene expression · Biotransformation

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Introduction

The biotransformation of nitriles by bacteria is of great importance in both the industrial production of various chemicals (e.g. acrylamide, acrylic acid, nicotinamide) and degradation of nitrile pollutants, mainly herbicides, in the environment. Nitrile hydrolysis, resulting in the production of the corresponding acids, proceeds in bacteria by two different enzymatic pathways: one-step transformation of nitriles by nitrilases or two-step reactions requiring nitrile hydratases to produce the corresponding amides, and amidases transforming the amides to carboxylic acids. Nitrile hydratases were classified, according to their cofactor, into Fe-type and Co-type families (for review see Martínková et al. 2010). The genes encoding the enzymes involved in nitrile transformation have been found in various bacteria, particularly in the genera *Pseudomonas* (Nishiyama et al. 1991) and *Rhodococcus* (Martínková et al. 2010). Using a metagenomic

approach, nitrile hydratase genes encoding predominantly Co-type enzymes highly similar to those of pseudomonads and rhodococci were also detected in soil samples (Precigou et al. 2001).

Rhodococcus erythropolis A4, originally designated *R. equi* A4 (Martínková et al. 1995) and later reclassified according to the results of sequencing of 16S rRNA genes (Vejvoda et al. 2007), was found to exhibit nitrile hydratase and amidase activities (Martínková et al. 1995). Its nitrile hydratase, consisting of two different subunits, exhibited enantioselectivity towards substituted nitriles (Přepechalová et al. 2001). The *R. erythropolis* A4 genes encoding the α and β subunits of Fe-type nitrile hydratase (*nha1* and *nha2*) were cloned and sequenced (Kubáč et al. 2008). The enantioselective amidase of *R. erythropolis* A4 was found to transform aliphatic, aromatic, heterocyclic and alicyclic amides to the corresponding carboxylic acids. It was demonstrated in previous studies that the range of substrates of this amidase included e.g. isonicotinamide (Vejvoda et al. 2006), 2,6-pyridinedicarboxamide and 2-cyanopyridine-4-carboxamide (Vejvoda et al. 2007), intermediates of cyano-cyclitol hydrolysis (e.g. 1-carboxamido-2,3,4-trihydroxy-cyclo-5-hexene) (D'Antona et al. 2010) and benzamide analogues 2,6-dichlorobenzamide, 3,5-dichloro-4-hydroxybenzamide and 3,5-dibromo-4-hydroxybenzamide (Veselá et al. 2012). In these studies, the steric hindrances in the substrates were found to exhibit significant effects on the amidase activity. The enzyme was also able to hydrolyze the amidic group of a bulky substrate such as lysergamide, albeit at low rates (Martínková et al. 2000). In acyl transfer reactions, the highest acyltransferase activities of *R. erythropolis* A4 amidase were observed for benzamide, 3-toluamide, propionamide, butyramide, valeramide and cyclohexanecarboxamide (Vejvoda et al. 2011).

The strain *R. erythropolis* CCM2595 degrades phenol, hydroxybenzoate, *p*-chlorophenol, aniline and other aromatic compounds (Čejková et al. 2005). A host-vector system has been developed for this strain (Veselý et al. 2003) and used for characterizing the expression of the *catRABC* operon involved in catechol degradation (Veselý et al. 2007) and for the construction of recombinant strains, which efficiently degrade phenol in industrial wastewater (Zídková et al. 2013).

In this paper, we describe the cloning of the aldoxime–nitrile–amide-converting gene clusters from both *R. erythropolis* A4 and CCM2595 strains, analysis of the expression of the amidase and nitrile hydratase genes from *R. erythropolis* A4 and the application of its nitrile hydratase and amidase for the hydrolysis of various cyanohydrins and 2-hydroxyamides.

Materials and methods

Chemicals

(*R*,*S*)-2-Hydroxy-4-phenylbutyronitrile (**1a**), (*R*,*S*)-4-methylmandelonitrile (**2a**), (*R*,*S*)-*E*-2-hydroxy-3-pentenenitrile (**3a**), (*R*,*S*)-3-phenoxymandelonitrile (**4a**) and (*R*,*S*)-2-chloromandelonitrile (**5a**) were kindly donated by the Graz University of Technology, Graz, Austria (Prof. H. Griengl). Other chemicals were analytical grade and purchased from standard commercial suppliers.

Strains, plasmids and primers used

R. erythropolis A4 (formerly *R. equi* A4) (Martínková et al. 1995) and *R. erythropolis* CCM2595 (Čejková et al. 2005) were sources of aldoxime–nitrile–amide-converting gene clusters. *R. erythropolis* CCM2595 also served as a host for the recombinant plasmids. *Escherichia coli* DH5α (Hanahan 1985) was used for gene cloning and for heterologous expression of the *R. erythropolis* A4 amidase gene.

The *E. coli* vector pKSAC45 (Holátko et al. 2009) was used for manipulations within the *R. erythropolis* chromosome. The promoter-probe vector pEPR1 carrying the *gfp* reporter gene (Knoppová et al. 2007) served as a basis for constructing the promoter-containing plasmids pEPRPami (using primers PAMIF1 and PAMIR1) and pEPRPnha (using primers PNHA1F1 and PNHA1R1). The expression vector pEXT20 (Dykxhoorn et al. 1996) was used for constructing the plasmid pEXT20ami using primers FAMIE and RAMIP. Oligonucleotide primers are shown in Table 1.

Growth conditions

Escherichia coli was grown in LB medium at 37 °C. *R. erythropolis* strains were grown at 30 °C in LBP medium (van der Geize et al. 2001) or in the minimal

Table 1 Oligonucleotide primers

Primer	5′–3′ DNA sequence[a]	Purpose
PAMIF	GGGCGGCATTCGGCTACTTT	Cloning of P*ami*
PAMIR1	TTAGATCTGAGTGATGCCGTAATGCTTT	Cloning of P*ami*
PNHA1F1	GATCACCGGCAAGACCTT	Cloning of P*nha*
PNHA1R1	AAGGATCCCTTCCATGCTTCGACGTAA	Cloning of P*nha*
FAMIE	CCGAATTCATCAGGAGCACACTT	Cloning of *ami* gene
RAMIP	GTCTGCAGCGGTGGAATTTGTTGTG	Cloning of *ami* gene
A4AMIPR	GAGTGATGCCGTAATGCTTT	Primer extension
A4NHAPR	GTACCAATCCCTTACCGTC	Primer extension
NHA2RRT	CGCAGGCTCGAGGTAACCCT	RT-PCR analysis
AMIFRT	ATGATGATCACCGGCAAGACC	RT-PCR analysis
NHA1FRT	CCCCAGGGCGAATACATCGTGG	RT-PCR analysis

[a] Restriction sites for cloning within overhanging 5′-ends are underlined

salt medium BSB (diGeronimo and Antoine 1976) with glycerol (10 g/l) and NH_4NO_3 (15 mM) as the carbon and nitrogen source, respectively. Acetonitrile or acetamide (15 mM each) was used as a sole source of nitrogen instead of NH_4NO_3 for testing the induction of promoter and enzyme activities. The *R. erythropolis* strains were cultivated in 500 ml flasks containing 100 ml of the appropriate medium. The selection media contained kanamycin (Km; 30 μg/ml for *E. coli*; 200 μg/ml for *R. erythropolis*).

Purification of nitrile hydratase

The nitrile hydratase from *R. erythropolis* A4 was partially purified using ion exchange and hydrophobic interaction chromatography and maintained as described previously (Přepechalová et al. 2001; Kubáč et al. 2008).

DNA and protein techniques

DNA isolation, polymerase chain reaction (PCR), transformation of *E. coli*, DNA cloning, and DNA and protein analyses were done using the standard methods (Sambrook and Russel 2001). Plasmid DNA was isolated from *R. erythropolis* with a PureYield™ Plasmid Miniprep System (Promega). Genomic DNA from *R. erythropolis* was isolated as described previously (Treadway et al. 1999). *R. erythropolis* cells were transformed with plasmid DNA by electroporation (Veselý et al. 2003).

RNA isolation and primer extension analysis

The RNA from *R. erythropolis* cultures was isolated by phenol extraction as described by Eikmanns et al. (1994). Reverse transcription was performed with SuperScript III Reverse Transcriptase (Invitrogen) and the fluorescein-labeled primers A4AMIPR and A4NHAPR complementary to the *R. erythropolis* A4 chromosomal DNA sequences within the 5′ ends of the *ami* and *nha1* genes, respectively. The reverse transcript was run on an automatic ALF DNA Sequencer (Pharmacia Biotech) alongside the sequencing ladder generated with the same primer.

RT-PCR analysis

The total RNA isolated from *R. erythropolis* A4 cells was incubated with Turbo™ DNase (Ambion) to remove traces of contaminating DNA. The template RNA (1 μg) was then transcribed into complementary DNA (cDNA) using SuperScript III Reverse Transcriptase (Invitrogen) and primer NHA2RRT (nt 7283–7302 in GenBank sequence no. AM946017, reverse). Reverse transcription (RT) product (1 μl) was used in subsequent PCR reactions (in 25 μl) using GoTaq DNA polymerase (Promega). The primers AMIFRT (nt 5812–5832, forward) and NHA2RRT (reverse) were used to amplify the fragment AMI–NHA2 (1491 bp), whereas primers NHA1FRT (nt 6287–6308, forward) and NHA2RRT were used to amplify the fragment NHA1–NHA2 (1016 bp). PCR

products were analyzed by electrophoresis in a 1 % agarose gel using GelRed™ (Biotium) staining.

GFP fluorescence intensity measurements

The promoter activity was determined using transcriptional fusions with the *gfp*uv reporter in the promoter-probe vector pEPR1 (Knoppová et al. 2007). The cell suspensions were washed twice with phosphate-buffered saline (8.25 g Na_2HPO_4, 2.05 g NaH_2PO_4, 4 g NaCl, in 1,000 ml, pH 7.4) and the cells were disrupted by sonication. The fluorescence of the cell extract was measured with a Saphire2 spectrophotometer (Tecan, USA) (excitation wavelength, 397 nm; emission wavelength, 509 nm). Fluorescence intensity was expressed in arbitrary units per mg of proteins (AU/mg protein). Protein concentration was determined by Bradford assay.

Enzyme assays

Nitrile hydratase and amidase activities were assayed at 30 and 45 °C, respectively, in cell suspensions from 0.5 to 1.0 ml of *R. erythropolis* cell cultures, with shaking. The reaction mixtures (0.5 ml) contained 20 mM benzonitrile (nitrile hydratase assay) or 10 mM benzamide (amidase assay) in 50 mM Tris/HCl buffer (pH 7.5) and 5 % (v/v) methanol as co-solvent. Specific enzyme activities were expressed as units per 1 ml of cell suspension of $OD_{600} = 1$ (U/ml [OD = 1]).

Biotransformations of cyanohydrins

The reactions proceeded in cell suspensions of *R. erythropolis* A4 ($OD_{600} = 1$) at 30 °C with shaking. The reaction mixtures (1 ml) contained 5 mM substrate **1a**–**5a** in 54 mM Na/K phosphate buffer, pH 7.0, and 5 % methanol as co-solvent. Alternatively, the reactions were catalyzed by a partially purified nitrile hydratase from the same strain at 32 °C. The reaction mixtures (0.5 ml) contained 75 μg of protein, 54 mM Na/K phosphate buffer, pH 7.5, and 5 % methanol as co-solvent.

Analytical methods

The reactions were terminated after various time intervals by acidification (HCl) and the cells were removed by centrifugation. The supernatants were analyzed by HPLC. The concentrations of benzonitrile, benzamide and benzoic acid were determined by HPLC using a Chromolith Flash RP-18e column (Merck, 25 mm × 4.6 mm) with a mobile phase (20 % v/v acetonitrile, 0.1 % v/v H_3PO_4) at 2 ml/min. Nitriles **1a**–**5a** and their corresponding amides **1b**–**5b** and carboxylic acid **1c**–**5c** were analyzed by HPLC as described previously (Osprian et al. 2003).

Nucleotide sequence accession numbers

The nucleotide sequences of the *R. erythropolis* A4 and *R. erythropolis* CCM2595 *oxd–ami–nha* gene clusters were deposited in GenBank under accession nos. AM946017 and JQ023030, respectively.

Results

Cloning and sequencing of the aldoxime–nitrile–amide-converting gene clusters from *R. erythropolis* A4 and *R. erythropolis* CCM2595

Amplification of the DNA fragment covering parts of the nitrile hydratase genes (coding for its α- and β-subunits) was the first step in isolating the aldoxime–nitrile–amide-converting gene cluster from *R. erythropolis* A4. Degenerated primers, designed according to the conserved regions of the gene sequences encoding Fe-type nitrile hydratases in various *Rhodococcus* strains, and the total *R. erythropolis* A4 DNA as a template were used for the respective PCR reaction (Kubáč et al. 2008). The regions flanking the cloned parts of the *nha1* and *nha2* genes were isolated by the plasmid rescue technique (Veselý et al. 2007). The sequencing of these regions revealed the presence of nine open reading frames (ORFs) on the 9552-bp chromosomal region of *R. erythropolis* A4. This sequence was found to be homologous with those of *R. globerulus* A-4 (Xie et al. 2003) (99 % identity) and *R. erythropolis* PR4 (NCBI RefSeq NC_012490.1) (95 % identity). According to sequence similarity searches, four ORFs correspond to the *oxd*, *ami*, *nha1* and *nha2* genes coding for aldoxime dehydratase, amidase and α- and β-subunits of nitrile hydratase, respectively. Further four ORFs (designated *nhr1*, *nhr2*, *nhr3* and *nhr4*) most likely encode regulatory proteins whereas the function of the product of the last

ORF (ORFb) is unknown. All these ORFs are oriented in the same direction (Fig. 1).

The sequence of the 9552-bp chromosomal region of *R. erythropolis* A4 was used to design primers for amplification of the homologous regions of the *R. erythropolis* CCM2595 chromosome by PCR. The PCR fragments obtained were sequenced and the DNA sequence of 8635-bp of *R. erythropolis* CCM2595 chromosome containing eight ORFs (*oxd*, *nhr2*, *nhr1*, *ami*, *nha1*, *nha2*, *nhr3* and *nhr4*) was determined. The DNA sequences of these 8635-bp regions in *R. erythropolis* A4 and CCM2595 are highly similar to each other (96 % identical nucleotides). The deduced amino acid sequences of *oxd*, *nhr1*, *ami*, *nha1*, *nha2*, *nhr3* and *nhr4* gene products of both *R. erythropolis* A4 and CCM2595 strains vary between 96 and 100 % identity. According to bioinformatic analysis, the proteins encoded by the aldoxime–nitrile–amide-converting gene cluster of *R. globerulus* A-4 and *R. erythropolis* PR4 show also the same range of identity of amino acids with those of *R. erythropolis* A4. The only significant difference in the products of the *R. erythropolis* A4 and CCM2595 aldoxime–nitrile–amide-converting gene clusters is the size of the deduced *nhr2* gene product. Due to a shift of the reading frame, the deduced size of the Nhr2 protein from the CCM2595 strain is 122 amino acids (the same size as in *R. globerulus* A-4 and *R. erythropolis* PR4), whereas that from the A4 strain is only 109 amino acids. The amino acid sequences of the C-terminal parts (from the position 79) of the respective Nhr2 proteins are totally different for the same reason.

Transcriptional analysis

Mapping promoters of R. erythropolis A4 ami and nha1–nha2 genes

To analyze the transcriptional regulation of *R. erythropolis* A4 genes coding for amidase and nitrile hydratase, the intergenic DNA fragments encompassing the assumed promoter regions were cloned in the *E. coli–Rhodococcus* promoter-probe vector pEPR1. Both *E. coli* clones harboring the resulting constructs, pEPRPami (478-bp insert) or pEPRPnha (345-bp insert), exhibited green fluorescence in contrast to the control, i.e. cells with the empty vector pEPR1. *R. erythropolis* A4 cells transformed with pEPRPami produced fluorescence whereas *R. erythropolis* A4 transformed with pEPRPnha did not. No plasmids could be isolated from either *R. erythropolis* A4 Km^R transformants, probably due to plasmid rearrangements or integration into the *R. erythropolis* A4 chromosome. To test the promoter activity of the cloned DNA fragments in the *Rhodococcus* background, the plasmids pEPRPami and pEPRPnha were transferred to a suitable plasmid host strain, *R. erythropolis* CCM2595 (Veselý et al. 2003), showing a high level of DNA sequence similarity of the aldoxime–nitrile–amide-converting gene cluster to that of *R. erythropolis* A4. Promoter activity (assayed as green fluorescence) was observed in the respective transformants of *R. erythropolis* CCM2595 grown on the plates with complete medium. This observation suggested that active promoters are located upstream of the *ami* and *nha1* genes of *R. erythropolis* A4 (Fig. 1).

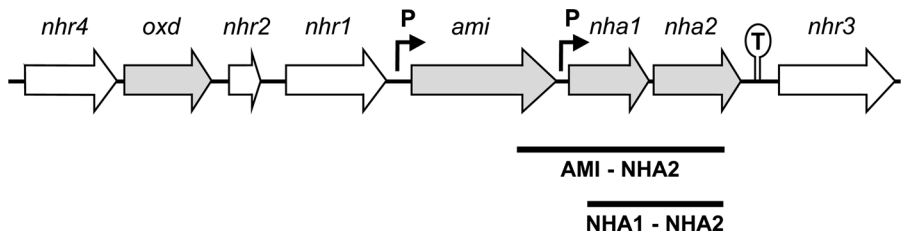


Fig. 1 Map of *R. erythropolis* A4 and *R. erythropolis* CCM2595 gene cluster involved in converting aldoximes, nitriles and amides. The genes encoding enzymes and putative regulators are shown as *grey* and *empty arrows*, respectively. The *bent arrows* represent the promoters of the *ami* and *nha* genes and the stem-looped structure (T) a putative terminator. The PCR fragments amplified from the cDNA template are shown as the *black lines* designated AMI–NHA2 and NHA1–NHA2. The ends of the *black lines* correspond to the location of the used primers (the sequences of the primers are shown in Table 1 and coordinates of their location on DNA sequence of *oxd–ami–nha* gene cluster published in GenBank are described in "Materials and methods" section)

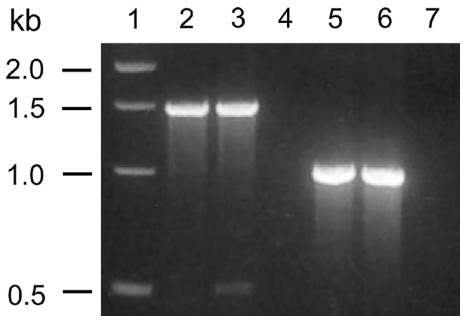


Fig. 2 RT-PCR analysis of the *ami–nha1–nha2* gene cluster. *Lane 1* 1 kb DNA ladder (New England Biolabs); *lanes 2*, *5* PCR products with chromosomal DNA as a template; *lanes 3*, *6* PCR products with cDNA as a template; *lanes 4*, 7 control PCR with RNA without reverse transcription as a template to exclude DNA contamination. The primers AMIFRT + NHA2RRT (*lanes 2*, *3*, *4*) or NHA1FRT + NHA2RRT (*lanes 5*, *6*, *7*) were used for the PCR reactions

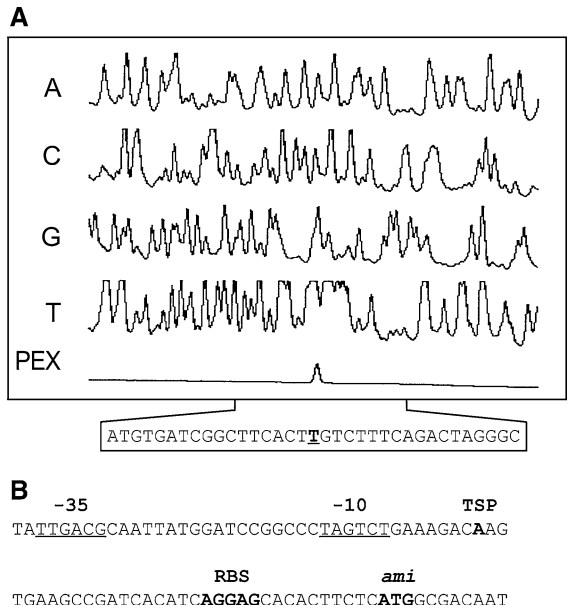


Fig. 3 **a** Determination of transcriptional start point (TSP) of *R. erythropolis* A4 *ami* gene. The *bottom peak* (PEX) represent cDNA synthesized in reverse transcription (primer extension) using RNA from *R. erythropolis* A4. The peaks generated by the sequencer represent the products of sequencing reactions (A, C, G, T) performed with the same fluorescein-labeled primer as that used for PEX. A portion of the nucleotide sequence derived from the sequencing signals is shown below. Note that the sequence is complementary to that shown in **b**. The TSP determined by PEX is in *bold* and *underlined.* **b** Nucleotide sequence of *R. erythropolis* A4 *ami* promoter region. The TSP, putative ribosome binding site (RBS) and initiation codon of the *ami* gene are in *bold*. The proposed −10 and −35 hexamers are *underlined*

Proof of cotranscription of the ami–nha1–nha2 genes

To verify that the *ami* gene is cotranscribed with the genes *nha1* and *nha2*, a PCR involving an RT step was carried out with RNA isolated from *R. erythropolis* A4 cells. As shown in Fig. 2, the DNA fragments of expected sizes (1491 and 1016 bp, respectively) were amplified using the primers AMIFRT + NHA2RRT complementary to the genes *ami* and *nha2* and the primers NHA1FRT + NHA2RRT complementary to the genes *nha1* and *nha2* when the same RT reaction product was used as a template. No PCR products were obtained when the RT step was omitted. These results indicate that a single transcript covering the all three genes, which thus form an operon, is produced. In addition, the separate transcript starting from an internal promoter upstream of the *nha1* gene was proved by a GFP reporter assay (Fig. 1).

Localization of ami and nha1 promoters

To localize the promoters of the *R. erythropolis* A4 *ami* (P*ami*) and *nha1* (P*nha*) genes, the transcriptional start points (TSPs) were determined by primer extension analysis using RNA isolated from *R. erythropolis* A4 grown in LBP medium. As shown in Fig. 3, the TSP was found 34 nt upstream of the translational start of the *ami* gene (Fig. 3b). The potential −35 and −10 promoter elements with an appropriate spacing (TTGACG, 18 nt, TAGTCT, 7 nt, TSP) were found closely upstream of the experimentally determined TSP. A weak TSP signal was observed 20 nt upstream of the translational start of the *nha1* gene (data not shown). The respective potential −35 and −10 promoter elements (ACAACA, 17 nt, CATGAT, 6 nt, TSP) thus represent the putative P*nha* promoter. Using the BLAST analysis, identical sequences covering the two promoter hexamers and the spacing region were found in sequences of the *ami–nha1* region of several *Rhodococcus* strains (e.g. *R. erythropolis* AJ270, *Rhodococcus* sp. N-771 and *Rhodococcus* sp. ACV2), whereas the sequences immediately upstream of the −35 region differed substantially. This supports the determination of TSP and the position of the promoter. Activities of the main operon promoter P*ami* and the internal promoter P*nha* were further analyzed.

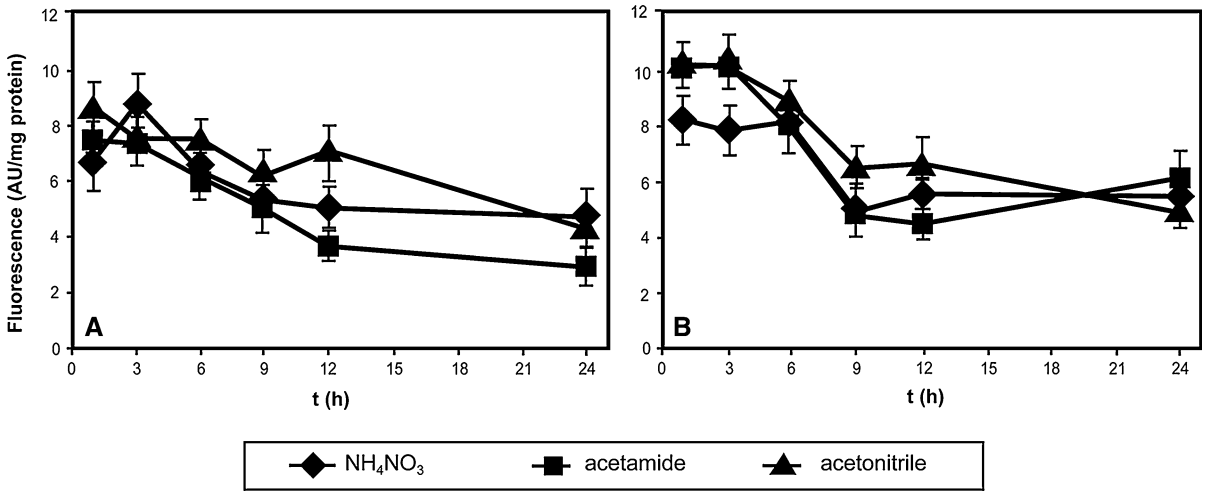


Fig. 4 Activity of *R. erythropolis* A4 P*ami* (**a**) and P*nha* (**b**) promoters in *R. erythropolis* CCM2595 cells harboring plasmid pEPRPami and pEPRPnha, respectively, grown on various nitrogen sources. Promoter activity was determined as the fluorescence intensity of the GFP reporter and expressed as arbitrary units per mg of proteins (AU/mg protein). NH_4NO_3, acetamide or acetonitrile were used as sole nitrogen sources. The values are averages from three independent measurements and the standard deviations are shown as *vertical bars*. The basal fluorescence of control *R. erythropolis* CCM2595 (pEPR1) cells was less than 3 AU/mg protein in all time points

Activity of the ami and nha promoters

To determine whether the transcription from the *R. erythropolis* A4 *ami* and *nha* promoters is inducible or constitutive, the activities of these promoters (transcriptionally fused to *gfp*uv in plasmids pEPRPami and pEPRPnha, respectively) were measured during batch cultivation in the cells of *R. erythropolis* CCM2595, harboring the respective plasmids, using the green fluorescence emitted by the GFP reporter. The inoculum for all cultures was grown in minimal BSB medium with NH_4NO_3 as a sole nitrogen source, whereas the cultures were cultivated in the same medium with various nitrogen sources (NH_4NO_3, acetamide and acetonitrile, respectively). Acetamide and acetonitrile were chosen as potential inducers according to the results of preliminary screening of 27 amides and nitriles (e.g. propionamide, acrylamide, cyclohexanecarboxamide, benzamide, propionitrile, butyronitrile, valeronitrile, benzonitrile) (data not shown). The growth rates of plasmid-harboring *Rhodococcus* strains were found to be higher on acetamide than on NH_4NO_3 or acetonitrile (data not shown). As shown in Fig. 4a, the activity of P*ami* was slightly higher on acetonitrile than on NH_4NO_3 or acetamide at almost all time points. The activity of P*nha* at the beginning of the cultivation (1–3 h) was significantly higher during growth on acetonitrile or acetamide than during growth on the inorganic nitrogen source (Fig. 4b). These results suggested that the P*ami* promoter activity is only weakly induced by acetonitrile, whereas P*nha* activity is moderately induced by both acetonitrile and acetamide at the beginning of the cultivation.

Amidase and nitrile hydratase activities and substrate specificities

The activities of amidase and nitrile hydratase in *R. erythropolis* A4 cells grown under various conditions were determined during batch cultivation. No significant differences in amidase (Fig. 5a) and nitrile hydratase (Fig. 5b) activities were observed between *R. erythropolis* A4 cultures grown on NH_4NO_3, acetonitrile or acetamide as a sole nitrogen source, respectively. The deletion derivative *R. erythropolis* A4 Δ*nhr1* lacking the functional *nhr1* gene, coding for a putative regulatory protein, exhibited the same activities of amidase and nitrile hydratase as the wild-type strain *R. erythropolis* A4 (data not shown).

The nitrile hydratase and amidase in *R. erythropolis* A4 were previously demonstrated to convert a wide range of various nitriles (Kubáč et al. 2008) but the transformations of cyanohydrins were not reported for

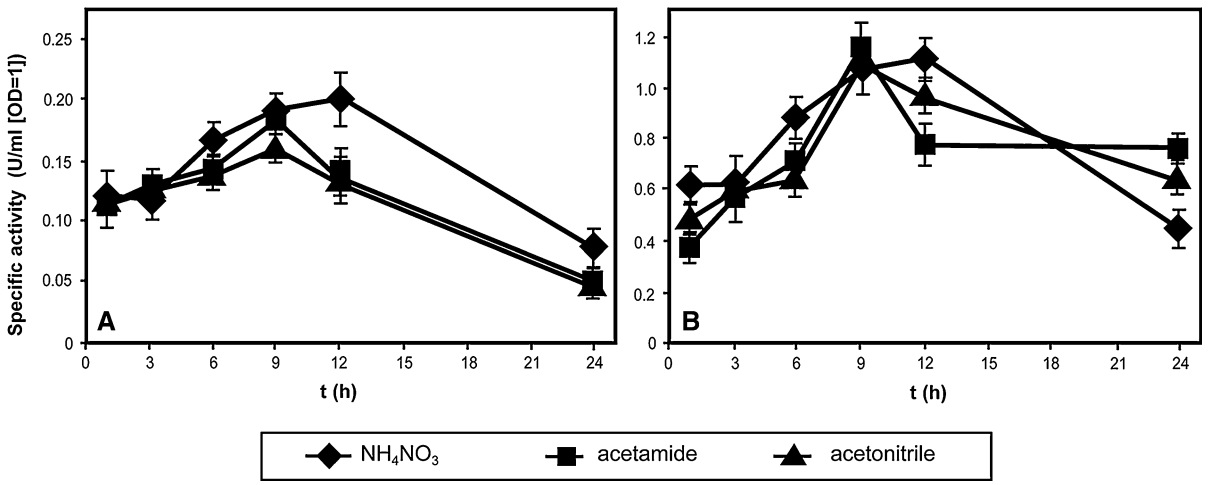
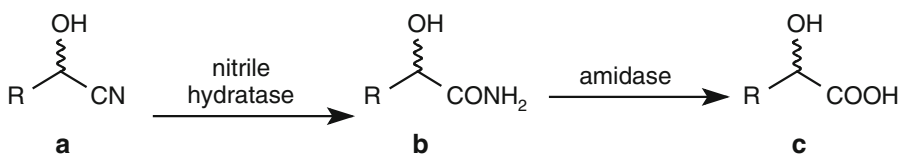


Fig. 5 Specific activity of amidase (**a**) and nitrile hydratase (**b**) in *R. erythropolis* A4 cellsgrown on various nitrogen sources. NH_4NO_3, acetamide or acetonitrile were used as sole nitrogen sources. The specific enzyme activities are expressed as units per 1 ml of cell suspension of $OD_{600} = 1$ (U/ml [OD = 1]). The values are averages from three independent assays and the standard deviations are shown as *vertical bars*

Table 2 Transformations of cyanohydrins by whole cells or by isolated nitrile hydratase of *R. erythropolis* A4



Substrate	*R*	Reaction time	Whole cells		Nitrile hydratase[a]
			Amide **b** (%)[b]	Acid **c** (%)[b]	Amide **b** (%)[b]
1a	2-Phenylethyl	10 min	89	7	92
		24 h	32	58	n.a.
2a	4-Methylphenyl	10 min	95	0	82
		24 h	71	18	n.a.
3a	*E*-Prop-1-enyl	10 min	41	0	31
		24 h	2	48	n.a.
4a	3-Phenoxyphenyl	10 min	95	1	62
		24 h	79	9	n.a.
5a	2-Chlorophenyl	10 min	92	0	69
		24 h	56	20	n.a.

n.a. not assayed

[a] Partially purified

[b] Analytical yield

these enzymes. Cyanohydrins are precursors of industrially important 2-hydroxy amides and 2-hydroxy acids such as (*R*)-2-chloromandelic acid used as a building block of Clopidogrel® (an antiplatelet agent) and 2-hydroxy-4-phenylbutyric acid used for synthesis of acetylcholin esterase inhibitors (Osprian et al. 2003). Therefore, a set of cyanohydrins were examined in this work. *R. erythropolis* A4 nitrile hydratase

transformed each of the tested cyanohydrins **1a**–**5a** to the corresponding 2-hydroxyamide (Table 2). This step proceeded at 89–95 % analytical yields within 10 min except for (*R*,*S*)-2-hydroxy-3-pentenenitrile (**3a**), which was partially lost by decomposition into aldehyde and HCN. The hydrolysis of the amides proceeded at much lower rates. After 24-h reactions, however, the yields of some of the corresponding acids were acceptable (Table 2). The total molar amount of products (amides and acids) after 24 h was by 10–24 % lower than the amount of substrates (cyanohydrins) added and by 6–16 % lower than the amount of products after 10-min reactions. This could be caused by further degradation of the acids or amides under the given conditions.

Heterologous expression of *R. erythropolis* A4 *ami* gene in *E. coli*

To ensure a highly efficient controlled expression of the *R. erythropolis* A4 *ami* gene, this gene was cloned into the *E. coli* expression vector pEXT20 under the strong P*tac* promoter, inducible by isopropyl-β-D-thiogalactopyranoside (IPTG). The resulting plasmid pEXT20ami was transferred into *E. coli* DHα cells and the 55-kDa protein (corresponding in size to *R. erythropolis* amidase) was detected by SDS-PAGE in lyzates of *E. coli* (pEXT20ami) grown in the presence of 0.5 mM IPTG for 6 h (Fig. 6a). The specific amidase activity in *E. coli* (pEXT20ami) reached its maximum value after growth under the same conditions (Fig. 6b).

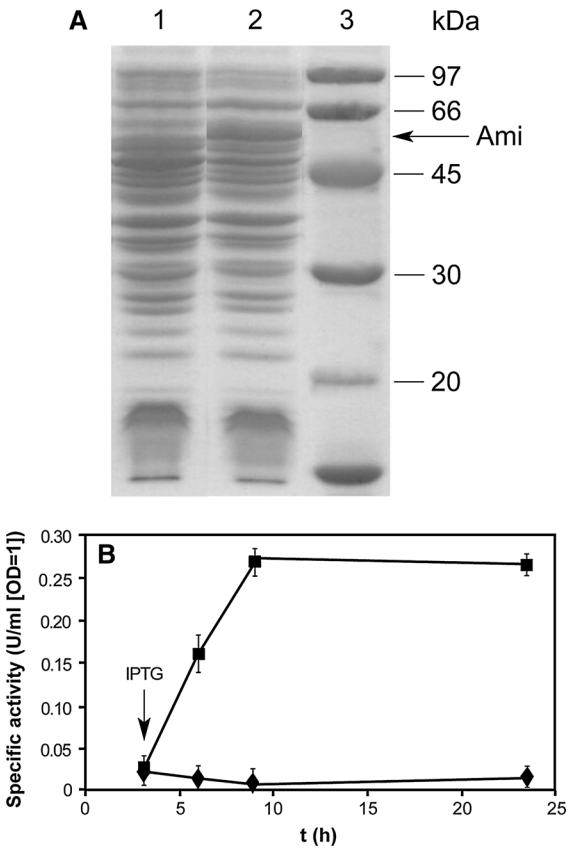


Fig. 6 **a** Proteins in lyzates of *E. coli* (pEXT20ami) cells (Coomassie blue-stained SDS-PAGE gel). *Lane 1* growth without induction; *lane 2* growth in presence of 0.5 mM IPTG for 6 h; *lane 3* protein size marker. The position of the induced amidase is indicated by an *arrow*. **b** Specific amidase activity in *E. coli* (pEXT20ami) cells grown without induction (*diamonds*) or after adding IPTG (final concentration 0.5 mM) 3 h after cultivation start (*squares*). The specific enzyme activities are expressed as units per 1 ml of cell suspension of $OD_{600} = 1$ (U/ml [OD = 1]). The values are averages from three independent experiments and the standard deviations are shown as *vertical bars*

Discussion

We cloned and sequenced the genes encoding the enzymes involved in nitrile biotransformation in the strain *R. erythropolis* A4, a Fe-type nitrile hydratase consisting of two subunits and an enantioselective amidase. These genes (*ami* and *nha1*, *nha2*) are part of the cluster of eight genes oriented in the same direction (Fig. 1). We also identified a homologous gene cluster in the strain *R. erythropolis* CCM2595. Using the sequence data from the NCBI database, we found the same organization of the eight genes involved in aldoxime, nitrile and amide metabolism in the genomes of *R. erythropolis* PR4, *R. erythropolis* SK121 and *R. qingshengii* BKS 20–40 (NCBI Reference Sequences NC_012490.1, NZ_ACNO01000109.1 and NZ_AODN01000096.1, respectively) and in *R. globerulus* A-4 (Xie et al. 2003).

RT-PCR analysis showed that a single transcript covering the *ami*, *nha1* and *nha2* genes, which thus form an operon, is produced. The separate transcript starting from an internal promoter upstream of the *nha1* gene was proved by a GFP reporter assay. The TSP of the *ami* gene was determined and the potential −35 (TTGACG) and −10 (TAGTCT) promoter hexamers of the P*ami* promoter were found in regular distance upstream. Similarly, TSP of the *nha1* gene and the relevant potential −35 (ACAACA) and −10

(CATGAT) promoter sequences were deduced. The hexamers of both promoters are similar to the defined consensus sequences of vegetative promoters of the closely related actinobacterium *Corynebacterium glutamicum* (Pátek and Nešvera 2011) and also to the key promoter sequences found in other *Rhodococcus* genes (Komeda et al. 1996; Veselý et al. 2007). We propose therefore, that the main promoter of the *ami–nha1–nha2* operon of *R. erythropolis* A4 (P*ami*) and the internal promoter P*nha* are recognized by a primary (vegetative) sigma factor of RNA polymerase.

Using the *gfp* reporter system, we found that the P*nha* promoter activity is moderately induced by both acetonitrile and acetamide at the beginning of the cultivation (Fig. 4b). An inducing effect of both acetonitrile and acetamide on nitrile hydratase activity was also observed in the *R. erythropolis* AJ270 strain encoding a two-component nitrile hydratase highly similar to that of *R. erythropolis* A4 (O'Mahony et al. 2005). A similar inducing effect of both acetonitrile and acetamide was described for the *R. erythropolis* AJ270 amidase (O'Mahony et al. 2005). The inducing effect of various nitriles and amides on the activity of *R. erythropolis* MP50 enantioselective amidase, exhibiting a low similarity to that of *R. erythropolis* A4, was also observed (Hirrlinger et al. 1996). However, we found that the *R. erythropolis* A4 P*ami* promoter was only weakly induced by acetonitrile (Fig. 4a). Since a substantial part of the promoter activity was not influenced by the induction, we can consider the *R. erythropolis* A4 *ami* and *nha1–nha2* transcription to be semi-constitutive.

Despite the fact that we have observed a moderate induction of *R. erythropolis* A4 amidase and nitrile hydratase gene expression by acetonitrile at the transcriptional level, we found no significant differences in the amidase and nitrile hydratase activities between *R. erythropolis* A4 cultures grown on NH_4NO_3, acetonitrile or acetamide as a sole nitrogen source, respectively. Similar disparity between the changes at the transcriptional level and enzyme activity was observed e.g. in several enzymes involved in amino acid biosynthesis in *C. glutamicum* (Glanemann et al. 2003).

The extent to what Fe-type nitrile hydratases and the enantioselective amidases from various rhodococci differ in their substrate specificities has not yet been clarified. The analysis of the amino acid sequences of the Fe-type nitrile hydratases of rhodococci indicated

Table 3 Abilities of *R. erythropolis* strains to hydrolyze various types of nitriles and amides

Substrate(s)	Strain				Reference(s)
	R312	AJ270	A4	NCIMB 11540	
Aliphatic nitriles	+	+	+[b]		Nagasawa et al. (1986), Meth-Cohn and Wang (1997), Přepechalová et al. (2001)
(Hetero)aromatic nitriles		+	+		Meth-Cohn and Wang (1997), Vejvoda et al. (2007), Veselá et al. (2012)
2-Hydroxynitriles[a]			+	+	Osprian et al. (2003), this work
2-Aminonitriles[a]		+		+	Wolf et al. (2001), Wang (2005)
3-Hydroxynitriles[a]	+	+	+		Wang (2005)
2-Arylpropionitriles		+	+		Wang (2005)
Alicyclic nitriles		+	+		Wang (2005)
3-Aminonitriles[a]	+		+	+	Winkler et al. (2005)
Cyano-cyclitols			+		D'Antona et al. (2010)
Isonicotinamide			+		Vejvoda et al. (2006)
Lysergamide			+		Martínková et al. (2000)

No entry means no data available

[a] Including their substituted analogues

[b] Production of amides by isolated nitrile hydratase

that these enzymes are highly conserved. However, their substrate specificities were only examined for some of them and, moreover, under different experimental conditions (Kubáč et al. 2008). Only a few Fe-type nitrile hydratases (from *R. erythropolis* R312, A4 and AJ270) were examined with various types of substrates (Table 3).

The amino acid sequence of *R. erythropolis* A4 amidase distinguishes it from all known amidases of rhodococci. The amidase from *R. globerulus* A-4, differing in three amino acids, is the most similar rhodococcal amidase (Xie et al. 2003), whereas the amidase from *Rhodococcus* sp. N-771 which has been characterized in most detail differs from the *R. erythropolis* A4 amidase in 19 amino acids (Ohtaki et al. 2010). It is possible that even this relatively small divergence could be responsible for their differing substrate specificities. The *R. erythropolis* A4 amidase was found to catalyze the conversion of various amides to practically important carboxylic acids such as 2-arylpropionic acids (Martínková et al. 1996), lysergic acid (Martínková et al. 2000), 3-amino acids (Winkler et al. 2005) or isonicotinic acid (Vejvoda et al. 2006) (Table 3). Some differences were observed in the substrate specificities of this enzyme and the amidase from the *R. erythropolis* R312 strain (with aa sequence identical to amidase from the N-771 strain) also in acyl transfer reactions (Fournand et al. 1998; Vejvoda et al. 2011). However, as with nitrile hydratases, there are insufficient data available to compare the substrate specificities of known amidases in more detail, since the enzymes have been examined with different substrates in most cases (Table 3).

In this work, the use of *R. erythropolis* A4 nitrile hydratase and amidase for the transformation of various cyanohydrins was examined. The same set of cyanohydrins was previously also tested with whole cells of *R. erythropolis* NCIMB 11540 (Osprian et al. 2003). With some reservations (since the reaction conditions were different) we propose that the relative activities for various cyanohydrins differ in these two strains. For instance, 3-phenoxymandelonitrile, was efficiently converted by the A4 strain, whereas the rate of its conversion by the NCIMB 11540 strain was ten times lower than that of 2-hydroxy-4-phenylbutyronitrile. In addition, the reaction rates of all cyanohydrin conversions were significantly lower in NCIMB 11540 than in A4 (by ca. one order of magnitude). The relative activities of the amidase showed the same tendency in both strains. The enantioselectivities of both nitrile hydratase and amidase for cyanohydrins and the corresponding amides, respectively, seemed to be low, as demonstrated by the chiral HPLC analysis of the amide intermediates obtained from (*R*,*S*)-2-hydroxy-4-phenylbutyronitrile and (*R*,*S*)-2-chloromandelonitrile by the A4 strain (unpublished results). *R. erythropolis* A4 may thus be used for the enantioretentive hydrolysis of optically pure cyanohydrins rather than for the dynamic kinetic resolution of racemic cyanohydrins.

Acknowledgments This work was supported by grant LC06010 from the Ministry of Education, Czech Republic, by Grant P504/11/0394 from the Czech Science Foundation and by the institutional research concept RVO 61388971. We thank prof. H. Griengl (Graz University of Technology, Graz, Austria) for kindly providing the cyanohydrins as well as useful advice on their biotransformation and analysis and D. Lukavská for excellent technical assistance.

Conflict of interest The authors declare that they have no conflict of interest.

References

Čejková A, Masák J, Jirků V, Veselý M, Pátek M, Nešvera J (2005) Potential of *Rhodococcus erythropolis* as a bioremediation organism. World J Microbiol Biotechnol 21:317–321

D'Antona N, Nicolosi G, Morrone R, Kubáč D, Kaplan O, Martínková L (2010) Synthesis of novel cyano-cyclitols and their stereoselective biotransformation catalyzed by *Rhodococcus erythropolis* A4. Tetrahedron Asymmetry 21:695–702

diGeronimo MJ, Antoine AD (1976) Metabolism of acetonitrile and propionitrile by *Nocardia rhodochrous* LL100-21. Appl Env Microbiol 31:900–906

Dykxhoorn DM, St Pierre R, Linn T (1996) A set of compatible *tac* promoter expression vectors. Gene 177:133–136

Eikmanns BJ, Thum-Schmitz N, Eggeling L, Ludtke KU, Sahm H (1994) Nucleotide sequence, expression and transcriptional analysis of the *Corynebacterium glutamicum gltA* gene encoding citrate synthase. Microbiology 140:1817–1828

Fournand D, Bigey F, Arnaud A (1998) Acyl transfer activity of an amidase from *Rhodococcus* sp. strain R312: formation of a wide range of hydroxamic acids. Appl Env Microbiol 64:2844–2852

Glanemann C, Loos A, Gorret N, Willis LB et al (2003) Disparity between changes in mRNA abundance and enzyme activity in *Corynebacterium glutamicum*: implications for DNA microarray analysis. Appl Microbiol Biotechnol 61:61–68

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

Hirrlinger B, Stolz A, Knackmuss HJ (1996) Purification and properties of an amidase from *Rhodococcus erythropolis* MP50 which enantioselectively hydrolyzes 2-arylpropionamides. J Bacteriol 178:3501–3507

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

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

Komeda H, Hori Y, Kobayashi M, Shimizu S (1996) Transcriptional regulation of the *Rhodococcus rhodochrous* J1 *nitA* gene encoding a nitrilase. Proc Natl Acad Sci USA 93:10572–10577

Kubáč D, Kaplan O, Elišáková V, Pátek M et al (2008) Biotransformation of nitriles to amides using soluble and immobilized nitrile hydratase from *Rhodococcus erythropolis* A4. J Mol Catal B Enzym 50:107–113

Martínková L, Olšovský P, Přepechalová I, Křen V (1995) Biotransformations of aromatic dinitriles using *Rhodococcus equi* cells. Biotechnol Lett 17:1219–1222

Martínková L, Stolz A, Knackmuss HJ (1996) Enantioselectivity of the nitrile hydratase from *Rhodococcus equi* A4 towards substituted (*R,S*)-2-arylpropionitriles. Biotechnol Lett 18:1073–1076

Martínková L, Křen V, Cvak L, Ovesná M, Přepechalová I (2000) Hydrolysis of lysergamide to lysergic acid by *Rhodococcus equi* A4. J Biotechnol 84:63–66

Martínková L, Pátek M, Schlosserová AB, Kaplan O, Uhnáková B, Nešvera J (2010) Catabolism of nitriles in *Rhodococcus*. In: Alvarez HM (ed) Biology of *Rhodococcus*. Springer Verlag, Berlin, pp 171–206

Meth-Cohn O, Wang M-X (1997) An in-depth study of the biotransformation of nitriles into amides and/or acids using *Rhodococcus rhodochrous* AJ270. J Chem Soc Perkin Trans 1(8):1099–1104

Nagasawa T, Ryuno K, Yamada H (1986) Nitrile hydratase in *Brevibacterium* R312. Biochem Biophys Res Commun 139:1305–1312

Nishiyama M, Horinouchi S, Kobayashi M, Nagasawa T, Yamada H, Beppu T (1991) Cloning and characterization of genes responsible for metabolism of nitrile compounds from *Pseudomonas chlororaphis* B23. J Bacteriol 173:2465–2472

O'Mahony R, Doran J, Coffey L, Cahill OJ, Black GW, O'Reilly C (2005) Characterisation of the nitrile hydratase gene clusters of *Rhodococcus erythropolis* AJ270 and AJ300 and *Microbacterium* sp. AJ115 indicates horizontal gene transfer and reveals an insertion of IS1166. Antonie Van Leeuwenhoek 87:221–232

Ohtaki A, Murata K, Sato Y, Noguchi K et al (2010) Structure and characterization of amidase from *Rhodococcus* sp. N-771: insight into the molecular mechanism of substrate recognition. Biochim Biophys Acta 1804:184–192

Osprian I, Fechter MH, Griengl H (2003) Biocatalytic hydrolysis of cyanohydrins: an efficient approach to enantiopure α-hydroxy carboxylic acids. J Mol Catal B Enzym 24–25:89–98

Pátek M, Nešvera J (2011) Sigma factors and promoters in *Corynebacterium glutamicum*. J Biotechnol 154:101–132

Precigou S, Goulas P, Duran R (2001) Rapid and specific identification of nitrile hydratase (NHase)-encoding genes in soil samples by polymerase chain reaction. FEMS Microbiol Lett 204:155–161

Přepechalová I, Martínková L, Stolz A, Ovesná M et al (2001) Purification and characterization of the enantioselective nitrile hydratase from *Rhodococcus equi* A4. Appl Microbiol Biotechnol 55:150–156

Sambrook J, Russel DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Treadway SL, Yanagimachi KS, Lankenau E, Lessard PA, Stephanopoulos G, Sinskey AJ (1999) Isolation and characterization of indene bioconversion genes from *Rhodococcus* strain I24. Appl Microbiol Biotechnol 51:786–793

van der Geize R, Hessels GI, van Gerwen R, van der Meijden P, Dijkhuizen L (2001) Unmarked gene deletion mutagenesis of *kst*D, encoding 3-ketosteroid Δ1-dehydrogenase, in *Rhodococcus erythropolis* SQ1 using *sacB* as counterselectable marker. FEMS Microbiol Lett 205:197–202

Vejvoda V, Kaplan O, Kubáč D, Křen V, Martínková L (2006) Immobilization of fungal nitrilase and bacterial amidase—two enzymes working in accord. Biocatal Biotrans 24:414–418

Vejvoda V, Šveda O, Kaplan O, Přikrylová V et al (2007) Biotransformation of heterocyclic dinitriles by *Rhodococcus erythropolis* and fungal nitrilases. Biotechnol Lett 29:1119–1124

Vejvoda V, Martínková L, Veselá AB, Kaplan O et al (2011) Biotransformation of nitriles to hydroxamic acids via a nitrile hydratase–amidase cascade reaction. J Mol Catal B Enzym 71:51–55

Veselá AB, Pelantová H, Šulc M, Macková M et al (2012) Biotransformation of benzonitrile herbicides via the nitrile hydratase–amidase pathway in rhodococci. J Ind Microbiol Biotechnol 39:1811–1819

Veselý M, Pátek M, Nešvera J, Čejková A, Masák J, Jirků V (2003) Host-vector system for phenol-degrading *Rhodococcus erythropolis* based on the *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

Wang M-X (2005) Enantioselective biotransformations of nitriles in organic synthesis. Top Catal 35:117–130

Winkler M, Martínková L, Knall AC, Krahulec S, Klempier N (2005) Synthesis and microbial transformation of β-amino nitriles. Tetrahedron 61:4249–4260

Wolf LB, Sonke T, Tjen KCMF, Kaptein B, Broxterman QB, Schoemaker HE, Rutjes FPJT (2001) A biocatalytic route to enantiomerically pure unsaturated α-H-α-amino acids. Adv Synth Catal 343:662–674

Xie SX, Kato Y, Komeda H, Yoshida S, Asano Y (2003) A gene cluster responsible for alkylaldoxime metabolism coexisting with nitrile hydratase and amidase in *Rhodococcus globerulus* A-4. Biochemistry 42:12056–12066

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