Nitrilase from *Pseudomonas fluorescens* EBC191: cloning and heterologous expression of the gene and biochemical characterization of the recombinant enzyme

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The gene encoding an enantioselective arylacetone nitrilase was identified on a 3.8 kb DNA fragment from the genomic DNA of *Pseudomonas fluorescens* EBC191. The gene was isolated, sequenced and cloned into the L-rhamnose-inducible expression vector pJOE2775. The nitrilase was produced in large quantities and purified as a histidine-tagged enzyme from crude extracts of L-rhamnose-induced cells of *Escherichia coli* JM109. The purified nitrilase was significantly stabilized during storage by the addition of 1 M ammonium sulfate. The temperature optimum (50 °C), pH optimum (pH 6.5), and specific activity of the recombinant nitrilase were similar to those of the native enzyme from *P. fluorescens* EBC191. The enzyme hydrolysed various phenylacetone nitriles with different substituents in the 2-position and also heterocyclic and bicyclic arylacetone nitriles to the corresponding carboxylic acids. The conversion of most arylacetone nitriles was accompanied by the formation of different amounts of amides as by-products. The relative amounts of amides formed from different nitriles increased with an increasing negative inductive effect of the substituent in the 2-position. The acids and amides that were formed from chiral nitriles demonstrated in most cases opposite enantiomeric excesses. Thus mandelonitrile was converted by the nitrilase preferentially to *R*-mandelic acid and *S*-mandelic acid amide. The nitrilase gene is physically linked in the genome of *P. fluorescens* with genes encoding the degradative pathway for mandelic acid. This might suggest a natural function of the nitrilase in the degradation of mandelonitrile or similar naturally occurring hydroxynitriles.

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INTRODUCTION

Nitrilases hydrolyse nitriles that contain an R-CN group to the corresponding carboxylic acids and ammonia. Several nitrilases have been found in different plants and micro-organisms. Nitriles are widely used intermediates in the chemical industry and there is considerable interest in the utilization of nitrilases for the chemo-, regio-, or enantioselective production of carboxylic acids from nitriles. One interesting application of nitrilases is the possibility of an enantioselective hydrolysis of racemic α -substituted nitriles because some optically active α -substituted carboxylic acids such as α -hydroxycarboxylic acids (e.g. mandelic acid), α -methylcarboxylic acids (e.g. 2-arylpropionic acids, pro-fenes) and α -aminocarboxylic acids (amino acids) are interesting products or potential precursors for the synthesis

of antibiotics and pharmaceuticals (Bunch, 1998; Martinková & Kren, 2002; Schulze, 2002).

Previously, Layh *et al.* (1992) isolated several bacterial strains with nitrilase activities from the environment. They were isolated from enrichment cultures using different arylacetone nitriles such as 2-methyl- or 2-ethylbenzylcyanide as sole sources of nitrogen. One of these strains, *Pseudomonas fluorescens* EBC191, was able to use different arylacetone nitriles (e.g. 2-phenylpropionitrile) as nitrogen sources and converted the nitriles to the corresponding α -substituted carboxylic acids. It was demonstrated that strain EBC191 synthesized a nitrilase, which converted *O*-acetoxy-mandelonitrile preferentially to *R*-acetoxy-mandelic acid (Layh *et al.*, 1992). The enzyme was subsequently partially purified, biochemically characterized, and the N-terminal and some internal amino acid sequences were determined (Moser, 1996; Layh *et al.*, 1998). This enzyme seems to possess some potential for the enantioselective production of carboxylic acids from racemic nitriles. In the present work, the nitrilase gene was identified in a genomic library of *P. fluorescens*

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EBC191, expressed in *Escherichia coli* and the recombinant protein biochemically characterized.

METHODS

Bacterial strains and culture conditions. The isolation and characterization of *P. fluorescens* EBC191 has been described previously (Layh *et al.*, 1992). Strain EBC191 was grown at 30 °C in a mineral medium according to Dorn *et al.* (1974), supplemented with glutamate (25 mM). All cloning experiments and plasmid preparations were carried out in *E. coli* JM109 (Vieira & Messing, 1982). *E. coli* strains were grown at 37 °C in 2 × YT liquid medium or on 2 × YT agar plates (Sambrook *et al.*, 1989) supplemented with 100 µg ampicillin ml⁻¹.

DNA preparation, DNA manipulation and cell transformation. Small-scale plasmid preparation was performed by the method of Kieser (1984). The isolation of genomic DNA and all DNA manipulations were carried out as described by Sambrook *et al.* (1989). All enzymes for DNA manipulation were purchased from Roche Diagnostics and used according to the manufacturer's suggestions. Cells of *E. coli* were transformed with plasmid DNA by the method of Chung *et al.* (1989).

Genomic library construction and screening for the nitrilase gene. The amino acid sequence HKKQYKV of the N-terminal part of the nitrilase (Moser, 1996) was used to design oligonucleotide S1061 (5'-CAC/T-AAG/A-AAG/A-CAG/A-TAC/T-AAG/A-GT-3'). The oligonucleotide was radiolabelled at its 5' end by use of T4 polynucleotide kinase and [γ -³²P]ATP (Sambrook *et al.*, 1989). Genomic DNA from *P. fluorescens* EBC191 was digested with *Sall*, loaded onto an agarose gel and electrophoresed at 10 V cm⁻¹ for 1 h. Fragments of 3–5 kb were isolated and ligated to *Sall*-digested plasmid pJOE890. Plasmid pJOE890 is a positive selection vector based on palindromic DNA sequences (Altenbuchner *et al.*, 1992). The plasmid genomic library was screened by colony lift with QIABRANE nylon membranes (Qiagen). The immobilized DNA was hybridized to the α -³²P-labelled oligonucleotide S1061. Plasmid DNA from minilysates of positive colonies was analysed by Southern hybridization (Sambrook *et al.*, 1989) to confirm the presence of the oligonucleotide sequence in the plasmid inserts.

Nucleotide sequence analysis. DNA sequences were determined by the dideoxy chain-termination method using an automated DNA sequencer (ALF-Sequencer, Pharmacia Biotech). Database searches were performed online with the programs BLASTX, BLASTP and BLASTN provided by the BLAST e-mail server (Altschul *et al.*, 1990; Gish & States, 1993). CLUSTALW (Thomson *et al.*, 1994) was used to align the amino acid sequences. All parameters were set at their default values. The sequence alignments were edited and analysed by using the multiple sequence alignment editor and shading utility GeneDoc (version 1.1.004) (Nicholas & Nicholas, 1996).

Amplification of the nitrilase gene by PCR. The forward primer P1 (5'-GAA-ATT-CCA-TAT-GAC-GGT-GCA-TAA-AAA-ACA-G-3') and the reverse primer PfluHisB (5'-CGG-GAT-CCC-TTG-TCG-CCT-TGC-TCT-TCT-3') were used to amplify a 1052 bp fragment containing the nitrilase gene. To facilitate cloning of the PCR product, *Nde*I and *Bam*HI restriction sites were added to the primers. The PCR product was cut with *Nde*I and *Bam*HI and inserted into the *Nde*I/*Bam*HI-cleaved plasmid vector pJOE2775 (Voff *et al.*, 1996) to give pIK9. The native stop codon was replaced by (CAT)₆TGA, which encodes a C-terminal His₆ tag. All PCR reaction mixtures contained 10–100 ng genomic DNA in a volume of 40 µl, 0.5 µM of each forward and reverse primer (MWG-Biotech), 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTP (Pharmacia Biotech) and 2.5 U *Taq* DNA polymerase

(Pharmacia Biotech). The PCR reactions were performed in a PTC-200 thermal cycler (MJ Research). The PCR protocol comprised an initial denaturation for 1 min at 94 °C, followed by 30 cycles of denaturation for 1 min at 92 °C, annealing of primers for 1 min at 50 °C and extension for 2 min at 72 °C, with extension for 5 min during the last cycle. The PCR products were separated by electrophoresis using 1% (w/v) agarose gels at 10 V cm⁻¹ and DNA bands were stained with ethidium bromide.

Inverse PCR. Inverse PCR was performed as described by Ochman *et al.* (1993). To isolate the 5' flanking sequence of the chromosomal DNA region of plasmid pDC5, chromosomal DNA of strain EBC191 was cut with *Pvu*II and hybridized against a 946 bp fragment of plasmid pDC5. A 2 kb fragment was identified corresponding to the 5' flanking sequence of the chromosomal fragment of pDC5. Chromosomal DNA of strain EBC191 was cut with *Pvu*II and ligated. The primers S2299 (5'-GGC-ACC-TCG-CCA-AAT-ACG-3') and S2302 (5'-ATC-AAG-GAC-AAT-ATC-CAG-G-3') were used for PCR with the ligated DNA as template. The 5' flanking fragment of the chromosomal DNA region was cloned into *Eco*RV-cut pUC18 and sequenced.

Purification of the nitrilase. Cells of *E. coli* JM109(pIK9) were grown overnight in 2 × YT medium with 100 µg ampicillin ml⁻¹. The cells were subsequently diluted (1:100) in 600 ml fresh medium. Rhamnose (0.2%, w/v) was added to induce the nitrilase gene when the OD₆₀₀ of the cell cultures reached about 0.25. The cells were harvested after 4–12 h by centrifugation, washed twice with 30 ml Tris/HCl buffer (20 mM, pH 7.5) and resuspended in 30 ml of the same buffer. The cells were disrupted using a French press (16 000–20 000 p.s.i., 110–138 MPa) and the homogenate was centrifuged at 15 000 g for 30 min. The supernatant (soluble protein fraction) was used for further experiments. Since the recombinant nitrilase carried a C-terminal affinity tag of six consecutive histidine residues, it was purified using the QIAexpress nickel-nitrilotriacetic acid (Ni-NTA) protein purification system (Qiagen). The method is based on the interaction of positively charged metal ions such as Ni²⁺ to histidine residues. Spin columns were packed with 1 ml Ni-NTA agarose and the protein purification was done with about 30–45 mg soluble cell protein (about 10–15 mg nitrilase) per ml Ni-NTA agarose. The columns were subsequently washed with Tris/HCl buffer (20 mM, pH 7.5) plus 100 mM NaCl and 1 mM DTT and with Tris/HCl buffer (20 mM, pH 7.5) plus 150 mM NaCl, 1 mM DTT and 50 mM imidazole. Finally, the nitrilase was eluted with Tris/HCl buffer (20 mM, pH 7.5) plus DTT (1 mM), NaCl (100 mM) and 200 mM imidazole.

Enzyme assays. The nitrilase activity of resting cells was determined in reaction mixtures (1 ml each) containing 50 µmol Tris/HCl buffer (pH 7.5), 10 µmol nitrile and an appropriate amount of cells. The assays with the purified nitrilase contained in addition 1 µmol DTT and 1 µg bovine serum albumin (BSA). The assays with naphthalene-2-acetonitrile, naproxennitrile or ketoprofennitrile contained in addition 20% (v/v) methanol in order to increase the solubility of the substrates. All nitrile stock solutions (200 mM each) were prepared in methanol. The reaction mixtures were incubated at 30 °C. After different time intervals, samples (200 µl each) were taken and the reactions were stopped by the addition of 1 M HCl (20 µl). The samples were centrifuged at 15 000 g for 10 min and the supernatants were analysed using HPLC. The phenylglycinonitrile decomposes slowly to benzaldehyde, HCN and ammonia. At the same time the cyanohydrine mandelonitrile arises from the spontaneous reaction of benzaldehyde and HCN. In order to suppress the mandelonitrile side reaction by the nitrilase, the sampling intervals were shortened as much as possible by using a high concentration of nitrilase in the phenylglycinonitrile assay. The (*R*)-*O*-acetoxy-mandelonitrile and the conversion products decomposed to mandelonitrile and the corresponding conversion products even at

neutral pH. The arising mandelonitrile served as a competing substrate for the nitrilase reaction. Furthermore, the products of this side reaction were indistinguishable from the decomposition products of (*R*)-*O*-acetoxymandelic acid and amide. This problem was minimized by injecting the assay samples directly into the HPLC system without further treatment. Protein concentrations were determined using the Bio-Rad protein assay.

SDS-PAGE. The proteins were separated on a 9% (w/v) denaturing SDS-polyacrylamide gel using the discontinuous buffer system of Laemmli (1970) and stained with Coomassie blue R250.

Analytical methods. The different aromatic nitriles and their corresponding amides and acids were analysed by HPLC. A S1121 solvent delivery system equipped with a S3205 UV-VIS detector from Sykam was used. For the achiral analysis, a reversed-phase column [250 mm × 4 mm (internal diameter); GROM] filled with 5 µm diameter particles of Lichrospher RP18 (E. Merck) was used to identify individual compounds which were detected spectrophotometrically at 210 nm. Methanol (50% v/v) and H₃PO₄ (0.1%, v/v) in H₂O was used as the mobile phase to analyse the conversion of the different arylacetonitriles. Separation of the enantiomers of mandelic acid, 2-phenylpropionic acid and the corresponding amides was achieved on a Chiral-HSA column (ChromTech). The mobile phases consisted of sodium phosphate buffer (100 mM, pH 7.0) containing 4.5% (v/v) acetonitrile, or sodium phosphate buffer (10 mM, pH 6.0) plus 0.5% (v/v) 2-propanol, respectively. The separation of the enantiomers of 2-phenylglycine and 2-phenylglycineamide was achieved on a Crownpak CR+ column (Daicel Chemical Industries) at 38 °C with 10.3 g HClO₄ l⁻¹ (pH 1) plus 10% (v/v) methanol as mobile phase. The enantiomers of naproxen were separated on a Chiral-AGP column (ChromTech) with 10 mM sodium phosphate buffer (pH 7.0) as mobile phase. Aliphatic nitriles such as propionitrile, butyronitrile, isobutyronitrile, 2-methylbutyronitrile and valeronitrile and their corresponding acids were analysed by gas chromatography. The reaction mixtures (1 ml each) were acidified with 0.1 ml 1 M HCl and the substrates and products extracted into dichloromethane (0.2 ml). Aliquots of the organic phase (3 µl each) were injected into a gas chromatograph from Packard Instrument (model 437A), equipped with a flame-ionization detector with a FS-Hydrotex β-PM column (0.5 m × 0.25 mm, Macherey-Nagel) and a Merck chromatointegrator (model 2000, E. Merck). The column temperature was set between 80 and 130 °C; the injector and detector temperature were set at 220 and 250 °C, respectively.

Chemicals. All chemicals were obtained from Sigma-Aldrich Chemie or E. Merck. The biochemicals were supplied by Roche Diagnostics. The sources of α-acetoxymandelonitrile, naproxennitrile and ketoprofennitrile have been previously described (Bauer *et al.*, 1994; Layh *et al.*, 1992, 1994).

RESULTS

Cloning and sequence analysis of the nitrilase gene

A degenerate oligonucleotide (S1061) was derived from the N-terminal amino acid sequence of the purified nitrilase of strain EBC191 (Moser, 1996). The genomic DNA was cut with different restriction enzymes and hybridized against α-³²P-labelled oligonucleotide S1061 in order to test if oligonucleotide S1061 was specifically bound to genomic DNA fragments of strain EBC191. Genomic DNA treated with *Sall* resulted in a positive hybridization signal which corresponded to a DNA fragment of 3.8 kb (data not shown). Consequently, genomic DNA from strain EBC191 was digested with *Sall*, electrophoresed and fragments of 3–5 kb were ligated to *Sall*-digested plasmid pJOE890 (Altenbuchner *et al.*, 1992). This genomic plasmid library was screened with the labelled oligonucleotide S1061. Plasmid DNA from minilysates of two positive colonies (out of 1740 colonies) were further analysed by restriction analysis and Southern hybridization and the hybridization signal of one plasmid, designated pDC11, was confirmed. DNA sequence analysis revealed that plasmid pDC11 carried an inserted 3.8 kb *Sall* fragment which encoded a putative protein with a considerable sequence similarity (33–47% identical amino acids) to known nitrilases (Table 1). The putative nitrilase (*nitA*) of strain EBC191 consisted of 350 amino acid residues which corresponded to a predicted protein size of 37.7 kDa.

Heterologous expression of the nitrilase gene in *E. coli*

A 1052 bp DNA fragment containing the putative *nitA* gene of strain EBC191 was amplified from chromosomal DNA of strain EBC191 by PCR using the primer pair P1 and PfluHisB (see Methods). The PCR fragment was digested with *NdeI* and *BamHI* and inserted into *NdeI/BamHI*-cleaved plasmid vector pJOE2775 (Volff *et al.*, 1996), producing pIK9. In this plasmid the native stop codon of the nitrilase gene was replaced by the codons GGATTC-(CAT)₆TGA, which encoded the amino acids GlyPheHis₆ as C-terminus. *E. coli* JM109(pIK9) was grown in 2 × YT

Table 1. Sequence identity (% identical amino acids) of the nitrilase from *P. fluorescens* EBC191 to various nitrilases from different bacteria

The accession numbers in the SWISS-PROT and EMBL/DBJ/GenBank database are given as references.

	<i>Kpn</i>	<i>RrhJ1</i>	<i>RrhK22</i>	<i>Ctes</i>	<i>Afae</i>	<i>Pflu</i>	Accession no.
<i>Klebsiella pneumoniae</i> (<i>Kpn</i>)	100	39	33	30	31	37	J03196
<i>Rhodococcus rhodochrous</i> J1 (<i>RrhJ1</i>)		100	47	34	36	45	D11425
<i>Rhodococcus rhodochrous</i> K22 (<i>RrhK22</i>)			100	33	33	39	D12583
<i>Comamonas testosteroni</i> (<i>Ctes</i>)				100	30	33	L32589
<i>Alcaligenes faecalis</i> JM3 (<i>Afae</i>)					100	47	D13419
<i>Pseudomonas fluorescens</i> EBC191 (<i>Pflu</i>)						100	AY885240

medium to the early exponential growth phase (OD_{600} 0.25) and the production of the nitrilase induced for 4 h with 0.2 % (w/v) L-rhamnose. As a control, *E. coli* JM109-(pJOE2775) without *nitA* was cultivated under the same conditions and both cell extracts were compared using SDS-PAGE. In *E. coli* JM109(pIK9) an additional protein of the predicted size was observed (Fig. 1a). The SDS-PAGE analysis suggested that the nitrilase constituted about 20 % of the soluble proteins. The cell extracts were also analysed for nitrilase activity and it was observed that cell extracts from *E. coli* JM109(pIK9) converted 2-phenylpropionitrile with a specific activity of 1.1 U mg^{-1} . In contrast, the cell extract of the control strain did not show any nitrilase activity.

Purification and stabilization of the recombinant nitrilase

The nitrilase was purified from cell extracts prepared from rhamnose-induced cells of *E. coli* JM109(pIK9). Due to the C-terminal His₆ affinity tag, the recombinant nitrilase could be easily purified using the QIAexpress Ni-NTA protein purification system (see Methods). In this way, the His-tagged nitrilase was purified in a single step from cell extracts by imidazole metal affinity chromatography almost to homogeneity (Fig. 1b).

Upon storage, the activity of the purified enzyme was reduced to approximately 25 % of the initial activity after 14 days at 8 °C in the elution buffer (pH 7.5) or Tris/HCl

buffer (pH 7.5 ± 1 mM DTT). Less than 10 % of the initial activity was recovered after storage of the purified enzyme in the same buffer systems at -20°C . Therefore, the effects of different additives which are known for their protein-stabilizing properties were tested. The addition of glycerol (30 %, v/v), Tween (0.3 %, v/v), sucrose (1 mg ml⁻¹), NaCl (100 mM) or ammonium sulfate (1 M) decreased the rate of inactivation of the nitrilase during storage at -20°C (Table 2). The addition of ammonium sulfate resulted in an almost complete stabilization of the nitrilase. In this way, the purified enzyme could be stored at -20°C for several months without any significant loss of activity.

Effect of temperature and pH

The temperature optimum of the nitrilase was between 50 and 55 °C when the reaction was analysed for 10 min, or between 45 and 50 °C when the reaction time was 30 min (Fig. 2a). At temperatures below 40 °C the reaction rates were constant for 30 min. Above 40 °C the activity of the nitrilase decreased within the 30 min of the test. No enzyme activity was observed at 65 °C (Fig. 2a). The temperature optimum of the *P. fluorescens* EBC191 nitrilase resembled those reported for the nitrilases from various organisms, such as *Alcaligenes faecalis* strains JM3 (45 °C, 10 min conversion time; Nagasawa *et al.*, 1990) and ATCC 8750 (40–45 °C, 30 min conversion time; Yamamoto *et al.*, 1990).

The pH value was varied from 5 to 8.5 in three different buffer systems (Tris/HCl, sodium phosphate and citrate/succinate). The EBC191 nitrilase showed a broad pH optimum, with only small variations in enzyme activity (<10 %) between pH 5.5 and 7.5 (Fig. 2b). The activity strongly decreased at pH values below 5.5. The broad pH optimum of the EBC191 nitrilase was strikingly different

Fig. 1. Heterologous production and purification of the nitrilase from *P. fluorescens* EBC191. (a) Heterologous expression of the nitrilase gene. SDS-PAGE (12%) of crude extracts from *E. coli* JM109(pJOE2775) (vector control, lanes 2, 3) and JM109(pIK9) (*nitA* gene, lanes 4, 5) from rhamnose-induced (+) or uninduced (-) cells (15 µg crude extract per lane). The position of the nitrilase is indicated by an arrow. (b) Purification of the nitrilase via imidazole metal-affinity chromatography. Five micrograms of purified enzyme was analysed in a 9% polyacrylamide gel (lane 2). Molecular mass standard (M, lane 1) was obtained from Bio-Rad. The sizes and positions of the marker proteins are indicated on the left.

Table 2. Stabilization of the purified nitrilase from *P. fluorescens* EBC191 during storage by the addition of different compounds

The purified nitrilase (protein concentration 0.03 mg ml⁻¹) was incubated in 0.1 M Tris/HCl (pH 7.5) in the presence of various additives. The specific activity of the enzyme was analysed after five freeze-thaw cycles over a period of 14 days. The enzyme without any additive was used as a control. The activity in the presence of glycerol (3.57 U mg⁻¹) was set to 100 % and used as reference value.

Additive	Relative activity (%)
No additive	3.4
DTT (5 mM)	0.3
Imidazole (100 mM)	0
Glycerol (30 % v/v)	100
Tween 20 (0.3 % v/v)	56.4
Sucrose (1 mg ml ⁻¹)	44.2
NaCl (100 mM)	98.1
(NH ₄) ₂ SO ₄ (1 M)	228.8

Fig. 2. Effect of temperature and pH on the activity of the nitrilase from strain EBC191. (a) Temperature optimum. The reactions were performed for 30 min at various temperatures and 2-phenylpropionitrile was used as substrate. Samples were taken at 5 (■), 10 (▲) and 30 (●) min. (b) pH optimum. The reactions were carried out for 30 min at 30 °C in Tris/HCl (●), sodium phosphate (■) and citrate-succinate (▲) buffers (all 0.1 M). The maximum activity which was achieved under different temperature (9.66 U mg⁻¹) and pH (5.03 U mg⁻¹) conditions was set to 100% and used as reference value.

from other nitrilases, which in most cases demonstrate rather narrow pH optima at slightly alkaline pH values (Kiziak, 1998).

Substrate specificity

The conversion of benzonitrile, different substituted phenylacetoneitriles and aliphatic nitriles was examined (Table 3). The enzyme showed high relative activities with (substituted) phenylacetoneitriles, such as 2-phenylvaleronitrile (230 U mg⁻¹), phenylacetoneitrile (68 U mg⁻¹), mandelonitrile (2-hydroxyphenylacetoneitrile) (33 U mg⁻¹) and 2-phenylpropionitrile (4.1 U mg⁻¹) as substrates. The α -aminonitrile 2-phenylglycinonitrile was also an effective substrate. The corresponding amino acid phenylglycine was produced with high specific activities (26 U mg⁻¹). In contrast, (*R,S*)-*O*-acetylmandelonitrile and the optically pure (*R*)-*O*-acetylmandelonitrile were only converted to the corresponding acid with specific activities of ≤ 2 U mg⁻¹. The nitrilase also converted heterocyclic arylacetoneitriles, such as 2-thiopheneacetoneitrile and 3-thiopheneacetoneitrile. The differences in the turnover rates of these two substrates suggested a pronounced influence of the position of the heteroatom on the enzyme activity (Table 3). Indole-3-acetonitrile was hydrolysed to the plant growth hormone indole-3-acetic acid (7.2 U mg⁻¹). More hydrophobic substrates such as 1- and 2-naphthaleneacetoneitrile were hydrolysed with lower but clearly detectable activities (0.45 and 0.55 U mg⁻¹, respectively). In contrast, structurally similar substrates with an additional α -methyl group, such as naproxennitrile [2-(6-methoxy-2-naphthyl)propionitrile] and ketoprofennitril [2-(3-benzoylphenyl)propionitrile], were very poor substrates. The enzyme also converted benzonitrile and short-chain aliphatic nitriles with low specific activities (Table 3).

Enantioselectivity of the formation of the acids

The nitrilase demonstrated, depending on the substrates, an *R*- or *S*-selectivity (Table 3). The reactions were analysed

routinely after about 30% conversion for the sake of comparison. (*R,S*)-Mandelonitrile was hydrolysed preferentially to *R*-mandelic acid with an enantiomeric excess of 31%, whereas 2-phenylpropionitrile was mainly converted to the corresponding *S*-acids with enantiomeric excesses of 65%. The nitrilase showed a similar preference for the naproxennitrile, which also has a methyl group in the α -position (enantiomeric excess of 70% after 1% conversion). The conversion of 2-phenylglycinonitrile (2-aminophenylacetoneitrile) produced *L*-phenylglycine (*S*-2-aminophenylacetic acid) with an enantiomeric excess of 35%.

Amide production

The conversion of most nitriles by the nitrilase resulted not only in the formation of the corresponding carboxylic acids but also in the release of the corresponding amides (Table 3). The highest proportions of amides were obtained with racemic *O*-acetylmandelonitrile and the optically pure (*R*)-*O*-acetylmandelonitrile as substrates (43% and 28% of the substrates converted to the amides, respectively). The non-acetylated substrate mandelonitrile was converted to mandelic acid and mandelic acid amide with a product ratio of 80:20. The conversion of 2-phenylpropionitrile, phenylacetoneitrile, 2-phenylglycinonitrile, thiophene- and naphthylacetoneitriles resulted in the formation of considerably lower relative amounts of the corresponding amides (Table 3). The chiral HPLC analysis of the reactions demonstrated that the formation of the amides was to a certain degree enantioselective. Surprisingly, it was found that the acids and amides that were formed from chiral nitriles demonstrated in most cases opposite enantiomeric excesses. Thus, mandelonitrile was converted by the nitrilase preferentially to *R*-mandelic acid and *S*-mandelic acid amide (Table 3).

Genomic region of the nitrilase gene

Several additional putative ORFs were identified on the 3.8 kb *SalI* fragment surrounding *nitA*. Upstream of *nitA*,

Table 3. Substrate specificity of the nitrilase from *P. fluorescens* EBC191

The reactions were performed at 30 °C using the standard reaction protocol (see Methods). The enzyme activity for the formation of 2-phenylpropionic acid was set as 100 % (corresponding to 4.1 U mg⁻¹). The enantiomeric excesses (e.e.) are given as e.e. values (%) of the acids or amides formed after hydrolysis of about 30 % the respective nitriles (in the case of naproxennitrile after 1 % conversion). In the case of amide formation, the proportion of the amide in relation to the total amount of acid plus amide formed is given. —, Not relevant; NA, not analysed.

Substrate	Relative activity – acid formation (%)	e.e. value of acid (%)	Amide production (% of total products)	e.e. value of amide (%)
2-Phenylpropionitrile	100	65 (S)	0.25	6 (R)
2-Phenylvaleronitrile	5600	NA	0	—
2-Phenylbutyronitrile	7	NA	0	—
Phenylacetoneitrile	1650	—	3.9	—
2-Phenylglycinonitrile	640	35 (S)	10.6	92 (S)
Mandelonitrile	800	31 (R)	19	85 (S)
O-Acetylmandelonitrile	50	NA	43	NA
(R)-O-Acetylmandelonitrile	27	—	28	—
3-Phenylpropionitrile	95	—	0.1*	—
Indole-3-acetonitrile	175	—	0.1	—
2-Thiopheneacetonitrile	5000	—	1.5*	—
3-Thiopheneacetonitrile	850	—	1.5*	—
1-Naphthylacetoneitrile	11	—	0.25*	—
2-Naphthylacetoneitrile	13	—	0.25*	—
Naproxenenitrile	0.00095	70 (S)	0	—
Ketoprofenenitrile	0.0006	NA	0	—
Benzonitrile	0.25	—	0	—
Propionitrile	0	—	NA	—
Butyronitrile	<5	—	NA	—
Isobutyronitrile	<5	—	NA	—
2-Methylbutyronitrile	0	—	NA	—
Valeronitrile	100	—	NA	—

*No standards were commercially available for these amides, but in each case the formation of an additional peak was detected during the nitrile conversion by HPLC analysis, which showed a similar retention behaviour in relationship to the respective acids as observed for the well characterized acid/amide pairs.

ORF3 was identified which encoded a protein of 415 amino acid residues (43 773 Da) that showed weak homologies to indole-3-acetamide hydrolases such as IaaH from *Agrobacterium tumefaciens* (Barker *et al.*, 1983), which participates in the synthesis of the plant hormone indole-3-acetic acid from indole-3-acetonitrile. Further upstream of ORF3, the 3' end of an ORF was detected (corresponding to 113 amino acids at the C-terminus of the encoded protein) which showed homologies to transcriptional activators of the LuxR type. In order to obtain the complete sequence of this putative regulator, the region upstream of *nitA* was isolated via inverted PCR. Chromosomal DNA was cut with *PvuI*, self-ligated and the ligation mixture used for inverted PCR using the primers S2299 und S2302. Thus, a 2 kb PCR fragment was amplified and cloned into pUC18. One complete and one incomplete ORF were identified via sequence analysis and homology searches. At the 5' end of the isolated PCR fragment, a gene encoding a putative

aromatic permease was identified. The 41 amino acids encoded by this gene fragment (ORF1, Fig. 3, Table 4) shared 37 % (30 %) identical residues with PcaK (BenK) from *Pseudomonas putida* (*Acinetobacter* sp. ADP1), which are involved in the transport of aromatic compounds such as 4-hydroxybenzoate (Harwood *et al.*, 1994) and benzoate (Collier *et al.*, 1997). The amino acid sequence deduced from the DNA sequence of ORF2 indicated that the gene product consisted of 234 amino acids and had a molecular mass of 26 236 Da. The putative protein showed only a low degree of similarity to transcriptional activators of the LuxR type such as MoaR from *Klebsiella pneumoniae* (23 % identical residues, Azakami *et al.*, 1993) and CarR from *Erwinia carotovora* (18 % identical residues, McGowan *et al.*, 1995). A higher degree of sequence similarity was found in the C-terminal helix–turn–helix motif (Pabo & Sauer, 1984) which is responsible for the DNA binding of LuxR-like regulators.

Fig. 3. Genomic context of the nitrilase gene of strain EBC191.

The sequence analysis of the 3.8 kb genomic fragment of plasmid pDC11 showed an additional (incomplete) ORF downstream of the nitrilase gene. The deduced amino acid sequence of ORF5 demonstrated a high degree of homology (73 % identical amino acids) to a mandelate racemase from *P. putida* (Tsou *et al.*, 1990). In order to isolate the complete mandelate racemase (*mdIA*) gene and to analyse if strain EBC191 carried further mandelate pathway genes, plasmid pDC5 was isolated from the plasmid library constructed from the genomic DNA of strain EBC191 (see above) using the *nitA* gene as DIG-labelled DNA probe. Thus, plasmid pDC5 was obtained, containing 11 kb downstream of the *nitA* gene (Fig. 3). Sequence analysis and homology searches revealed that the deduced amino acid sequences of three ORFs (ORF5, ORF6, ORF7) downstream of the *nitA* gene were homologous to the enzymes of the mandelate pathway of *P. putida* (Tsou *et al.*, 1990). Therefore, strain EBC191 was tested for its ability to use mandelic acid as sole carbon

source and it was found that the strain grew on mineral media which were supplemented with *S*- or/and *R*-mandelic acid.

DISCUSSION

The nitrilase from *P. fluorescens* EBC191 is clearly homologous to previously described nitrilases. The highest degree of amino acid sequence identity (47 %) was observed with a nitrilase from *Alc. faecalis* strain JM3 (Table 1). Both enzymes preferentially converted arylacetoneitriles and showed only significantly lower (or no) activities with aliphatic nitriles or benzonitrile (Mauger *et al.*, 1990; Nagasawa *et al.*, 1990). The nitrilase from *Alc. faecalis* JM3 has been described as arylacetoneitrilase because of its substrate specificity, and it appears reasonable to also classify the nitrilase from *P. fluorescens* EBC191 as an arylacetoneitrilase. The conversion rates of the EBC191 nitrilase for phenylacetoneitriles

Table 4. Amino acid (aa) sequence homologies of the identified ORFs

ORF	No. of aa	Homologous proteins	Identity (%)	Putative function	GenBank accession no.
ORF1	>41	PcaK (<i>P. putida</i>)	37	Aromatic permease	U10895
		BenK (<i>Acinetobacter</i> sp. ADP1)	30		AF009224
ORF2	234	MoaR (<i>E. aerogenes</i>)	23	Transcriptional activator	D49928
		CarR (<i>E. carotovora</i>)	18		AF041840
ORF3	415	Iaah (<i>Agr. tumefaciens</i> , <i>Agr. rhizogenes</i>)		Indole-3-acetamide hydrolase	
			27		AB025110
			26		M61151
ORF4	350	NitA (<i>Alc. faecalis</i> JM3, <i>R. rhodochrous</i> J1, <i>K. pneumoniae</i>)		Nitrilase	
			47		D13419
			45		D11425
			37		J03196
ORF5	359	MdIA (<i>P. putida</i>)	73	Mandelate racemase	J05293
ORF6	397	MdIB (<i>P. putida</i>)	78	<i>S</i> -Mandelate dehydrogenase	J05293
ORF7	>41	MdIC (<i>P. putida</i>)	52	Benzoylformate decarboxylase	J05293

decreased in the order 2-phenylvaleronitrile > phenylacetonitrile > mandelonitrile > phenylglycinonitrile > 2-phenylpropionitrile > 2-acetylmandelonitrile > 2-phenylbutyronitrile (Table 2). Thus, there was no obvious correlation between the size or the polarity of the substituents and the enzymic activity. The extraordinarily high activity of the enzyme with the sterically rather demanding substrate 2-phenylvaleronitrile was remarkable because *P. fluorescens* EBC191 was originally obtained from an enrichment with 2-phenylbutyronitrile (α -ethylbenzylcyanide) as sole source of nitrogen (Layh *et al.*, 1992). This enrichment substrate was converted by the purified nitrilase with less than 1 % of the activity found with 2-phenylvaleronitrile. This was an unexpected observation because it has been previously suggested that enrichments with specific nitriles usually result in the isolation of bacteria with nitrile-hydrolysing enzyme systems demonstrating high activities towards the nitriles used as enrichment substrates (Layh *et al.*, 1997). The reason for the clear preference of the enzyme for the conversion of 2-phenylvaleronitrile is currently not clear. It was found that from all of the tested aliphatic nitriles the enzyme demonstrated a clear preference for valeronitrile as substrate, which was hydrolysed with a specific activity of approximately 4 U mg⁻¹. These results indicate that not only the presence and position of the aromatic ring but also the type of the alkyl side chain is a relevant factor which distinguishes suitable substrates.

Although the nitrilase from strain EBC191 converted various sterically hindered phenylacetoneitriles and also some bicyclic and heterocyclic arylacetoneitriles, some severe steric restriction was also evident which hindered the performance of the catalysis. While the activities observed with 1- and 2-naphthaleneacetoneitrile were about 10 % of the activity with 2-phenylpropionitrile, naproxennitrile was only converted with less than 1 % of the activity found with the naphthaleneacetoneitriles. The decrease in activity is probably not caused by the additional 6-methoxy group attached to the naphthalene ring system but by the combined effects of the extended ring system and the methyl group at the 2-position of the naproxennitrile molecule.

A potential drawback for the utilization of nitrilases in biotransformation reactions is the generally observed low stability of nitrilases in cell-free preparations. Also in this respect, the purified nitrilase from strain EBC191 clearly resembled other nitrilases. Thus, the arylacetoneitrilase from *Alc. faecalis* strain JM3 lost about 60 % of its activity after 1 week storage at 4 °C in potassium phosphate buffer (pH 7.0) in the presence of 1 mM DTT (Nagasawa *et al.*, 1990). The enzyme from *P. fluorescens* lost about 75 % of its initial activity after 2 weeks storage under similar conditions. Also the nitrilases from *Rhodococcus rhodochrous* NCIMB 11215, *R. rhodochrous* NCIMB 11216, *Fusarium* species and *Arthrobacter* sp. strain J1 were rapidly inactivated during storage (Bandyopadhyay *et al.*, 1986; Harper, 1976, 1977, 1985; Goldlust & Bohak 1989). Several investigators have added reducing agents such as DTT to the

enzyme preparations in order to prevent oxidation of the thiol residues which are supposed to be involved in the hydrolysing reaction (Kobayashi *et al.*, 1992; Nagasawa *et al.*, 1990). Furthermore, the addition of glycerol or ethylene glycol was used to avoid denaturation of highly purified enzymes from *R. rhodochrous* J1 and K22, *Arthrobacter* sp. strain J1, *Alc. faecalis* JM3 and ATCC 8750 during long-term storage at -20 °C (Kobayashi *et al.*, 1989, 1990; Nagasawa *et al.*, 1990; Bandyopadhyay *et al.*, 1986; Yamamoto *et al.*, 1992). Previously, a stabilizing effect of a combination of ammonium sulfate and glycerol was described for the nitrilase from *R. rhodochrous* J1. This effect was explained by the high salt concentrations which were supposed to prevent the dissociation of the nitrilase subunits (Nagasawa *et al.*, 2000). The presence of glycerol also significantly increased the stability of the purified enzyme from strain EBC191. An even more pronounced stabilizing effect was obtained in the presence of ammonium sulfate (1 M) (Table 1). Under these conditions, storage of the enzyme for several months at -20 °C and also repeated freeze-thawing cycles are possible almost without loss of enzyme activity. This treatment allows an almost unlimited storage of the purified nitrilase of *P. fluorescens*, which significantly facilitates the utilization of the enzyme.

The nitrilase from strain EBC191 produced significant amounts of amides from different substituted phenylacetoneitriles. The formation of amides has also been previously reported for other nitrilases from different sources. A nitrilase from *Fusarium oxysporum* released the amides (4–5 % of the respective products) largely independent of the structure of the nitriles converted (Goldlust & Bohak, 1989). The nitrilase from *Rhodococcus* sp. strain ATCC 39484 produced the amide (2 %) only during conversion of phenylacetoneitrile (Stevenson *et al.*, 1992). Furthermore, a ricinine-specific nitrilase from *Pseudomonas* sp. released the corresponding amide (7–10 % of the amount of the acid formed) and two nitrilases from the plant *Arabidopsis thaliana* hydrolysed 2-fluoroacetoneitriles (AtNit1) or β -cyano(L)-alanine (AtNit4) to significant amounts of the corresponding amides (Hook & Robinson, 1964; Robinson & Hook, 1964; Effenberger & Osswald, 2001; Osswald *et al.*, 2002; Piotrowski *et al.*, 2001). The nitrilase from strain EBC191 demonstrated a clear correlation between the structure of the substrates and the relative proportion of the respective amides formed. Thus it was evident that large amounts of the amides were formed from *O*-acetoxymandelonitrile, mandelonitrile, and phenylglycinonitrile (43, 20 and 8 %, respectively). In contrast, significantly lower relative amounts of the amides were formed from the other substrates tested. The negative inductive (-I) effect at the 2-position of the substrates tested increased from 2-phenylvaleronitrile (C₃H₇ group) to 2-phenylbutyronitrile (C₂H₅ group), 2-phenylpropionitrile (CH₃ group), phenylacetoneitrile (H atom), 2-phenylglycinonitrile (NH₂ group), mandelonitrile (OH group) and *O*-acetylmandelonitrile (*O*-acetyl group). This suggested that the synthesis of the amide intermediates increased with an increasing polarity

(increasing $-I$ effect) of the substituents at the 2-position, although it can not be excluded that other properties of the substituents might influence the degree of amide formation. A similar effect was recently described for the enzymic hydrolysis of 2-fluoroacetonitriles using the nitrilase AtNit1 from *Arabidopsis thaliana*. This enzyme converted simple aliphatic nitriles mainly to the corresponding acids. In contrast, 2-fluoroarylacetonitriles were preferentially converted to the amides and only minor amounts of the corresponding carboxylic acids were formed. It was suggested that the increased formation of the amides was due to the presence of highly electron-withdrawing substituents such as fluorine or nitro-groups (Effenberger & Osswald, 2001; Osswald *et al.*, 2002). The results of the present study substantiate this observation and demonstrate that it may be possible to utilize classical nitrilases with certain substrates as nitrile hydratases forming stoichiometric amounts of the corresponding amides.

The nitrilase gene was physically connected in the genome of *P. fluorescens* to the genes encoding several enzymes of the mandelate pathway. Previously the genes surrounding a nitrilase gene have only been studied in detail for *R. rhodochrous* J1 and *Bacillus* sp. OxB-1. It was found that the *R. rhodochrous* J1 gene for a transcriptional regulator (*nitR*) similar to *araC* from *E. coli* was located directly downstream of *nitA* (Komeda *et al.*, 1996). In contrast, the *Bacillus* sp. OxB-1 nitrilase gene was structurally connected to a gene encoding a phenylacetaldoxime dehydratase (Kato *et al.*, 2000). Thus on a molecular level the nitrilase organization was completely different in *P. fluorescens* compared to the Gram-positive strains previously studied: the nitrilase gene was physically connected to the mandelate pathway genes, the nitrilase converted mandelonitrile and the strain grew with mandelic acid. The ability of the *P. fluorescens* strain to grow with mandelonitrile (which could not be tested directly as growth substrate because of its rapid decomposition at neutral pH to benzaldehyde and cyanide) was supported by the following observations. Mandelonitrile is formed in nature from different cyanogenic glycosides which are synthesized by different plants in order to prevent feeding of insects. Thus it seems reasonable that the natural function of the nitrilase in *P. fluorescens* might be the utilization of these hydroxynitriles produced by plants in large quantities. A similar combination of the mandelate pathway with a functionally interrelated enzyme has recently been described for *P. putida* ATCC 12633. In this strain the genes of the mandelate pathway are connected to a mandelic acid amide hydrolase (McLeish *et al.*, 2003). It appears that in various pseudomonads an expanded mandelate pathway has a function in the metabolism of nitrogen containing derivatives of mandelic acid.

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