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Conversion of Sterically Demanding α,α-Disubstituted Phenylacetonitriles by the Arylacetonitrilase from *Pseudomonas fluorescens* EBC191

Stefanie Baum,[a] Dael S. Williamson,[b] Trevor Sewell,[b] and Andreas Stolz[a]

Institut für Mikrobiologie, Universität Stuttgart, Stuttgart, Germany,[a] and Electron Microscope Unit, University of Cape Town, Rondebosch, South Africa[b]

The nitrilase from *Pseudomonas fluorescens* EBC191 converted 2-methyl-2-phenylpropionitrile, which contains a quaternary carbon atom in the α-position toward the nitrile group, and also similar sterically demanding substrates, such as 2-hydroxy-2-phenylpropionitrile (acetophenone cyanohydrin) or 2-acetyloxy-2-methylphenylacetonitrile. 2-Methyl-2-phenylpropionitrile was hydrolyzed to almost stoichiometric amounts of the corresponding acid. Acetophenone cyanohydrin was transformed to the corresponding acid (atrolactate) and amide (atrolactamide) at a ratio of about 3.4:1. The (*R*)-acid and the (*S*)-amide were formed preferentially from acetophenone cyanohydrin. A homology model of the nitrilase suggested that steric hindrance with amino acid residue Tyr54 could impair the binding or conversion of sterically demanding substrates. Therefore, several enzyme variants that carried mutations in the respective residues were generated and subsequently analyzed for the substrate specificity and enantioselectivity of the reactions. Enzyme variants that demonstrated increased relative activities for the conversion of acetophenone cyanohydrin were identified. The chiral analysis of these reactions demonstrated peculiar reaction kinetics, which suggested that the enzyme variants converted the nonpreferred (*S*)-enantiomer of acetophenone cyanohydrin with a higher reaction rate than that of the (preferred) (*R*)-enantiomer. Recombinant whole-cell catalysts that simultaneously produced the nitrilase from *P. fluorescens* EBC191 and a plant-derived (*S*)-oxynitrilase from cassava (*Manihot esculenta*) converted acetophenone plus cyanide at pH 4.5 to (*S*)-atrolactate and (*S*)-atrolactamide. These recombinant cells are promising catalysts for the synthesis of stable chiral quaternary carbon centers from ketones.

Organic nitriles are important synthons in the chemical industry because they are easily prepared and allow a facile extension of carbon chains. Therefore, a significant interest exists in biological systems that generate or convert nitriles, because these enzymatic processes might allow chemo-, stereo-, and/or enantioselective reactions, which are often difficult to perform in classical chemical processes. The most versatile enzymes in this respect are oxynitrilases, nitrilases, nitrile hydratases, and amidases (Fig. 1). Oxynitrilases (hydroxynitrile lyases) catalyze the addition of HCN to aldehydes and ketones forming α-hydroxynitriles (cyanohydrins) or the reverse reaction. Nitrilases and nitrile hydratases convert nitriles by the addition of water. Nitrilases are able to directly hydrolyze nitriles to the corresponding carboxylic acids, and nitrile hydratases convert nitriles to the amides, which then can be hydrolyzed by amidases to the corresponding carboxylic acids.

The chemo-, regio-, and enantioselectivities of oxynitrilases, nitrilases, nitrile hydratases, and amidases have been successfully applied for various biotransformation processes (10, 18, 28, 50). The usefulness of these enzymes for the chemical industry is also emphasized by several established large-scale industrial processes. The conversion of acrylonitrile to acrylamide by nitrile hydratases is quantitatively one of the most important biotransformation processes, and it was recently estimated that worldwide, about 400,000 tons per year of acrylamide is produced by biocatalytic processes (26, 57).

The applicability of nitrile-converting enzymes is still severely restricted because nitrilases and nitrile hydratases generally demonstrate only a low degree of enantioselectivity, and different approaches have been suggested in order to solve this problem. Thus, for (substituted) mandelonitrile(s), extensive screenings resulted in the identification of nitrilases that convert racemic (substituted) mandelonitrile(s) with a high degree of enantioselectivity to (*R*)-mandelic acid(s) (19, 26, 53), but these nitrilases demonstrate a high degree of enantioselectivity, specifically only with (substituted) mandelonitrile(s), and an analogous system for the synthesis of the (*S*)-enantiomers has never been described (37). A possible solution for this problem has recently been achieved by constructing "bienzymatic cascade reactions," which couple a highly (*S*)-specific plant-derived oxynitrilase with a bacterial nitrilase either *in vitro* (as immobilized enzyme catalysts) or *in vivo* (in recombinant whole-cell catalysts). These systems convert benzaldehyde plus cyanide efficiently to (*S*)-mandelic acid and/or (*S*)-mandeloamide (29, 40, 41). These biotransformation routes are interesting, as (*R*)- and (*S*)-mandelic acids are utilized in various chemical syntheses as convenient precursors for the introduction of a chiral center, which possess the extra advantage of bearing a useful functional group. Many mandelic acid derivatives also act as chiral auxiliaries for the introduction of a chiral center in stereoselective transformations (8).

Oxynitrilases as well as nitrilases convert a broad range of substrates (10, 28). Therefore, it might be expected that a combination of enantioselective oxynitrilases with bacterial (or fungal) nitrilases (or nitrile hydratases) should result in the generation of

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Address correspondence to Andreas Stolz, andreas.stolz@imb.uni-stuttgart.de.

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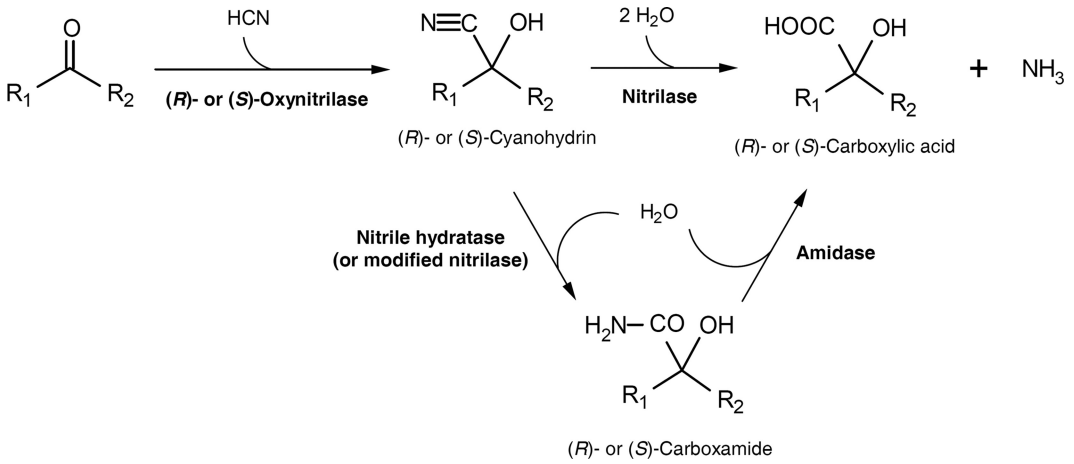


FIG 1 Schematic representation of the reactions catalyzed by oxynitrilases, nitrilases, nitrile hydratases, and amidases.

broadly applicable biocatalysts with the ability to convert a wide range of aldehydes (and ketones) plus cyanide to chiral 2-hydroxycarboxylic acids or 2-hydroxycarboxamides, which are important intermediates in chemical synthesis (8). Previously performed experiments aimed exclusively for the formation of (*S*)-mandelic acid and/or (*S*)-mandeloamide from benzaldehyde and cyanide (29, 40, 41), but there exist several other relevant targets for this kind of biotransformation. A very interesting group of products is α-alkyl-α-hydroxycarboxylic acids (and amides), which can be synthesized by a combination of oxynitrilases and nitrilases from different ketones and cyanide (Fig. 2). A "bienzymatic approach" is considered to be especially useful for the conversion of aromatic ketones, because oxynitrilases often do not quantitatively convert these substrates, as the reaction equilibrium is largely on the side of the ketones (5, 7, 15, 49). Furthermore, it was recently stated that the great challenge in the area of enantioselective enzyme-catalyzed cyanohydrin synthesis is the same challenge that the transition metal catalysts face: the efficient, high-yielding, and highly enantioselective conversion of ketones (17). Thus, it is reasonable to assume that the presence of a second (enantioselective) enzyme, such as a nitrilase, might result in the establishment of an efficient cascade reaction, which could circumvent the incomplete conversion of (aromatic) ketones by shifting the equilibrium to the product side. The interest in this type of reaction is further substantiated because (chiral) α-alkyl-α-hydroxycarboxylic acids (and amides) are interesting products or intermediates for the chemical synthesis of various alkaloids, antibiotics, insect pheromones, neurotransmitter agonists and antagonists, vitamins, prostaglandin analogues, and other chiral agricultural and pharmaceutically active and also natural products (9, 12, 34, 39, 44).

The intended reaction sequence requires that nitrilases be available to convert sterically demanding nitriles with four non-hydrogen substituents attached to the α-position of the nitrile molecule. In the present paper, the ability of a nitrilase to convert these types of substrates is demonstrated and applied for the construction of whole-cell catalysts that allow the "one-pot synthesis" of optically active sterically hindered 2-hydroxycarboxylic acids.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The construction of plasmids pIK7, pIK9, and pDHE22, which encode the nitrilases from *Alcaligenes faecalis* ATCC 8750, *Pseudomonas fluorescens* EBC191, and *Synechocystis* sp. strain PCC6803, respectively, was described previously (16, 20, 21). The gene for the rhodococcal nitrilase was amplified from a nitrile-converting *Rhodococcus rhodochrous* strain. The encoded protein was >97% identical to the previously described nitrilase from *Rhodococcus rhodochrous* J1 (23). The rhodococcal gene was cloned into the same pBR322-derived vector system (pJOE2775) (48) as that used for the other nitrilases. The genes were expressed under the control of a rhamnose-inducible promoter (43). *Escherichia coli* JM109 was used as the host strain for all plasmids. The cultivation and induction conditions for the recombinant strains used were described previously (16, 20, 40).

Enzyme assays. The induced cells (culture volumes of 10 to 25 ml) were harvested in the early stationary growth phase by centrifugation (5,000 rpm for 15 min at 4°C), washed in Tris-HCl buffer (50 mM; pH 7.5), and resuspended in 1 to 5 ml 50 mM Na-citrate (pH 4.5) or Na/K-phosphate buffer (pH 7). Subsequently, the cells were diluted in the reaction buffers to appropriate cell densities. The nitrilase activity of the resting cells was routinely determined in reaction mixtures (1 ml each) containing 50 to 100 mM Na-citrate (pH 4.5) or Na/K-phosphate buffer (pH 7) plus a 10 mM concentration of the respective nitrile (from 0.05 to 0.2 M stock solutions in methanol). The reaction mixtures were incubated at 30°C in a thermomixer at 1,100 rpm. After different time intervals, samples (90 μl each) were taken, and the reactions were stopped by the addition of 1 M HCl (10 μl). The cells were removed by centrifugation at

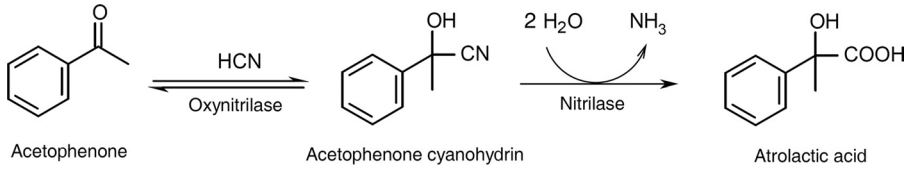


FIG 2 Enzymatic synthesis of 2-hydroxy-2-phenylpropionic acid (atrolactic acid) by a bienzymatic reaction cascade.

13,500 rpm for 10 min, and the supernatants were analyzed by using high-performance liquid chromatography (HPLC).

In certain experiments, the nitrilase activities were determined by the quantitation of the amount of ammonia that was enzymatically released from the nitriles. The reaction mixtures contained 50 mM KH-phthalate buffer (pH 5) and an appropriate amount of resting cells of *Escherichia coli* JM109(pIK9) (optical density at 600 nm [OD_{600}] of 5 to 100). The reactions were started by the addition of a 5 mM (2-acetyloxy-2-methylphenylacetonitrile) or 10 mM (all other substrates) concentration of the respective nitrile (from 0.05 to 0.2 M stock solutions in methanol) to the mixtures. The reaction mixtures were incubated at 30°C in a thermomixer at 1,350 rpm. Aliquots were taken periodically (for 60 to 90 min; 5 to 9 per experiment), and the cells were removed by centrifugation (4°C at 13,500 rpm for 5 min). The reactions were quantified via the release of ammonia by using a commercial test kit, as suggested by the manufacturer of the test kit (Aquaquant; Merck). The observed rates of ammonia release were corrected for the small amounts of ammonia released by the cells in the absence of added nitriles.

Construction of nitrilase mutants. The amino acid exchanges at position 54 of the nitrilase from *P. fluorescens* EBC191 were introduced by using the QuikChange site-directed mutagenesis kit from Stratagene and degenerated oligonucleotides (Eurofins, Hamburg, Germany) with "NNS degeneracy" (where N is A/C/G/T and S is C/G). Thereby, all 20 proteinogenic amino acids could be obtained. Cells of *E. coli* XL1-Blue (Stratagene) were transformed with the mutated plasmids, and positive clones were selected on LB agar plates with ampicillin (100 μg/ml).

DNA preparation, DNA manipulation, transformation, PCR, DNA sequencing, database searches, and sequence alignments were performed as described previously (21). Plasmid DNA was custom sequenced by GATC Biotech (Konstanz, Germany).

Analytical methods. The turnover of the nitriles was routinely quantified by high-performance liquid chromatography (HPLC). The conversion of acetophenone cyanohydrin was analyzed by using a Lichrospher RP18 column (Trentec Analysentechnik, Rutesheim, Germany). Lichrospher RP8 columns (Trentec) were used for all other separations.

2-Phenylpropionitrile, mandelonitrile, and their corresponding amides and acids were analyzed by using a solvent system consisting of 40% (vol/vol) methanol and 0.3% (vol/vol) H_3PO_4 in H_2O and a flow rate of 0.6 ml/min.

The solvent system for the analysis of 2-methyl-2-phenylpropionitrile and its product was made up from 50% (vol/vol) acetonitrile plus 0.2% (vol/vol) H_3PO_4 in water using a flow rate of 0.9 ml/min.

2-Hydroxy-2-phenylpropionitrile (acetophenone cyanohydrin) and its products were analyzed by using a mobile phase consisting of 40% (vol/vol) acetonitrile and 0.2% (vol/vol) H_3PO_4 in H_2O (flow rate of 0.7 ml/min).

The separation of the enantiomers of mandelate, mandeloamide, 2-hydroxy-2-phenylpropionate (atrolactate), and 2-hydroxy-2-phenylpropionamide (atrolactamide) was achieved on a Chiral-HSA column (150 by 4 mm, with 5-μm particles; ChromTech AB, Hägersten, Sweden). The mobile phase (flow rate of 0.8 ml/min) for the separation of atrolactate and atrolactamide consisted of Na-phosphate buffer (25 mM; pH 7) containing 2% (vol/vol) 2-propanol as an organic modifier. The enantiomers of mandelic acid and mandeloamide were separated by using a solvent system containing 10 mM Na-phosphate buffer (pH 7) plus 4.5% (vol/vol) acetonitrile.

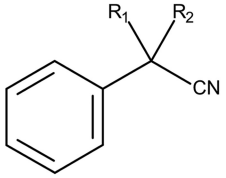
The separated compounds were generally detected by using a detector wavelength of 210 nm, and acetophenone cyanohydrin and its products were analyzed at 215 nm.

Homology modeling. Models were created by using methods outlined previously by Williamson et al. (51). GenTHREADER (31) was used to identify 12 homologues from the Protein Data Bank (PDB), and two of these were selected for the modeling of the arylacetonitrilase from *P. fluorescens* EBC191. The D chain of the beta-alanine synthase from *Drosophila melanogaster* (PDB accession number 2VHI) was selected as the best overall model because the sequence has the same features as those of the arylacetonitrilase with the exception of the N-terminal 67 amino acids, which were omitted from the template. In particular, the template reported under accession number 2VHI is unique among the structures of nitrilase homologues in providing a template for residues 238 to 247. However, the arylacetonitrilase has a 7-amino-acid insertion relative to the template reported under accession number 2VHI beginning at position 140. This important section of beta-sheet, which leads up to the active-site cysteine, was modeled on the basis of the coordinates of the amidase from *Nesterenkonia* sp. strain AN1 (accession number 3HKX). The amidase structure also has a tyrosine in the location homologous to Tyr54, which is hydrogen bonded to the homologously located active-site Glu137, thus providing added detail in the vicinity of the active site.

Identification of the enantiomers of atrolactate and atrolactamide. An enantiopure standard of (*S*)-atrolactate was commercially available (abcr GmbH, Karlsruhe, Germany), and a racemic standard of atrolactamide was synthesized (see below). The analysis of the chemically synthesized atrolactamide by chiral HPLC (conditions described above) demonstrated the presence of two signals (retention time [R_t] of 2.3 min and R_t of 3.8 min). In order to correlate these signals with the respective enantiomers, acetophenone plus cyanide were converted by *E. coli* JM109(pIK9)(pJOE5361.1), which simultaneously expressed the (*S*)-oxynitrilase from cassava (*Manihot esculenta*) and the nitrilase from *P. fluorescens* EBC191 and which had been successfully used for the production of (*S*)-mandelic acid and (*S*)-mandeloamide from benzaldehyde plus cyanide (40). Thus, it was found that during the reaction, the signal with an R_t of 3.8 min was formed preferentially, which was thus identified as (*S*)-atrolactamide.

Chemicals. 2-Hydroxy-2-phenylpropionitrile (acetophenone cyanohydrin) was synthesized basically according to a procedure described previously by Gassman and Talley (14), as follows. Acetophenone (0.1 mol) was mixed with 30 ml of dry dichloromethane. The round-bottom flask was put on ice, and 0.11 mol (11 g) trimethylsilylcyanide was slowly added. The mixture was stirred after the addition of about 10 mg of ZnI_2 for 6 h at room temperature. Finally, 50 ml HCl (20%) and 50 ml of tetrahydrofurane were added to split the silylether, and the reaction mixture was stirred for another 2 h on ice. The acetophenone cyanohydrin was extracted 3 times with diethyl ether (in total, about 100 ml). The ether phases were combined and washed with a saturated solution of sodium disulfite ($Na_2S_2O_5$) in order to extract the nonconverted acetophenone. Finally, the ether phase was dried over $MgSO_4$, and the ether was evaporated under reduced pressure. A yield of 14.2 g (97%) of acetophenone cyanohydrin was recovered. ^{1}H nuclear magnetic resonance (NMR) ($CDCl_3$, 250 MHz) δ 7.32 (d, 2H, Ph), 7.18 (m, 3H, Ph), 4.40 (br s, 1H, OH), 1.61 (s, 3H, Me); infrared (IR) (film) 3,420 (OH), 2,243 (CN) cm^{-1}.

Atrolactamide was synthesized from acetophenone cyanohydrin according to a method described previously by Zha et al. (56), as follows. A total of 40 mmol (6 g) acetophenone cyanohydrin was added to 70 ml cold concentrated HCl in a round-bottom flask. The mixture was saturated with HCl gas while being stirred on ice for 20 min. The reaction mixture was left on ice (without stirring) for 7.5 h. During this time period, the ice melted, and the temperature of the reaction mixture reached about room temperature. Subsequently, 200 ml of a cold NaOH solution (20%, wt/vol) and some solid pieces of NaOH were added slowly until a pH value of 9 was reached. The precipitated atrolactamide was extracted 3 times with 50 ml (each) of ethylacetate. The combined extracts were washed 2 times with water to remove the NaCl. Finally, the ethylacetate phase was dried by the addition of Na_2SO_4, and the ethylacetate was evaporated under reduced pressure. Atrolactamide was recrystallized from diethyl ether in a fridge overnight. The precipitated white crystals were collected and dried under reduced pressure in a desiccator. Thus, 2.8 g of atrolactamide (70% yield) was obtained. ^{1}H NMR (dimethyl sulfoxide [DMSO], 500 MHz) δ 7.53 (m, 2H, Ph), 7.3 to 7.23 (m, 4H, Ph and NH), 7.07 (s, 1H, NH), 5.91 (s, 1H, OH), 1.6 (s, 3H, Me); ^{13}C NMR (DMSO, 125 MHz) δ 177, 145,



R_1:	R_2:	
CH_3	H	2-Phenylpropionitrile
CH_3	CH_3	2-Methyl-2-phenylpropionitrile
CH_3	$OCOCH_3$	2-Acetyloxy-2-methylphenylacetonitrile
CF_3	OCH_3	2-Methoxy-2-trifluoromethylphenylacetonitrile
C_2H_5	C_2H_5	2-Ethyl-2-phenylbutyronitrile

FIG 3 Structural formulas of the nitriles used.

128, 127, 125, 75, 26; IR (KBr) 1,669 (C=O), 3,266 to 3,314 (NH), 3,453 (OH) cm^{-1}; mass spectrometry (MS), 165 (M).

2-Methyl-2-phenylpropionitrile and 2-methyl-2-phenylpropionic acid were purchased from Accela ChemBio Inc. (San Diego, CA), and 2-acetyloxy-2-methylphenylacetonitrile was kindly supplied by S. van Pelt, TU Delft (47). The sources of all other chemicals were described previously (41).

RESULTS

Release of ammonia from sterically demanding nitriles by the nitrilase from *P. fluorescens* EBC191. The nitrilase from *P. fluorescens* EBC191 was previously shown to hydrolyze a wide range of arylacetonitriles, such as 2-phenylpropionitrile (2-methylphenylacetonitrile), 2-phenylbutyronitrile, or 2-phenylvaleronitrile, and also α-hydroxynitriles, such as mandelonitrile, with rather high specific activities. This indicated that this nitrilase is especially well suited for the conversion of substrates carrying one bulky substituent at the α-carbon atom (4, 13, 20). Therefore, whole cells of *Escherichia coli* JM109(pIK9) (which synthesize the recombinant enzyme) were incubated with different sterically demanding nitriles carrying two bulky substituents at the α-carbon (for structural formulas of the nitriles used, see Fig. 3). The release of ammonia was determined by using a colorimetric assay. The nitrilase was able to release ammonia from the α,α-disubstituted phenylacetonitriles 2-acetyloxy-2-methylphenylacetonitrile and 2-methyl-2-phenylpropionitrile (with about 50% and 3%, respectively, of the rates observed with 2-phenylpropionitrile as substrate).

Stoichiometric conversion of 2-methyl-2-phenylpropionitrile to 2-methyl-2-phenylpropionic acid. The conversion of 2-methyl-2-phenylpropionitrile was subsequently analyzed by HPLC using a commercially available standard of the presumed reaction product 2-methyl-2-phenylpropionic acid. These experiments demonstrated that the resting cells indeed converted 2-methyl-2-phenylpropionitrile to almost stoichiometric amounts of 2-methyl-2-phenylpropionic acid (Fig. 4). The nitrilase hydrolyzed 2-methyl-2-phenylpropionitrile with about 8% of the reaction rates observed with 2-phenylpropionitrile (both reactions measured at pH 7). This demonstrated that the additional methyl group attached to the α-carbon atom resulted in a significant decrease in the reaction rates.

Comparison of the acid stabilities of different nitrilases. α-Hydroxynitriles (and also α-alkyl-α-hydroxynitriles) differ from nitriles, which carry only hydrogen and/or alkyl substituents in the α-position by their instability in neutral aqueous solutions. This occurs because α-hydroxynitriles exist under neutral conditions in equilibrium with their corresponding aldehydes (or ketones) and cyanide (3). The enantioselective biotransformation of α-hydroxynitriles requires, therefore, either acidic conditions or the presence of large amounts of organic solvents.

Thus, the stability of the α-alkyl-α-hydroxynitrile acetophenone cyanohydrin was analyzed at different pH values. These experiments demonstrated that at 30°C, a pH value of <5 was necessary to prevent a significant decay of acetophenone cyanohydrin. Furthermore, it was found that a decrease in the reaction temperature to 6°C had a stabilizing effect on the substrate (data not shown). A comparison of these results with previously performed experiments with mandelonitrile (38) demonstrated that under acidic conditions, mandelonitrile was more stable than acetophenone cyanohydrin. The stability experiments with acetophenone cyanohydrin suggested that the intended biotransformation reaction required pH values of <5 (at least at 30°C).

Cells of *E. coli* keep their cytoplasmic pH value in acidic environments above the pH value in the reaction medium. Nevertheless, the acidification of the medium can cause some decrease of the cytoplasmic pH value (36). Therefore, different recombinant *E. coli* strains that synthesized nitrilases from *P. fluorescens* EBC191, *A. faecalis* ATCC 8750, *Synechocystis* sp. PCC6803, or *Rhodococcus* sp. were compared with respect to the acid tolerances of their nitrile-converting systems. The whole-cell catalysts were

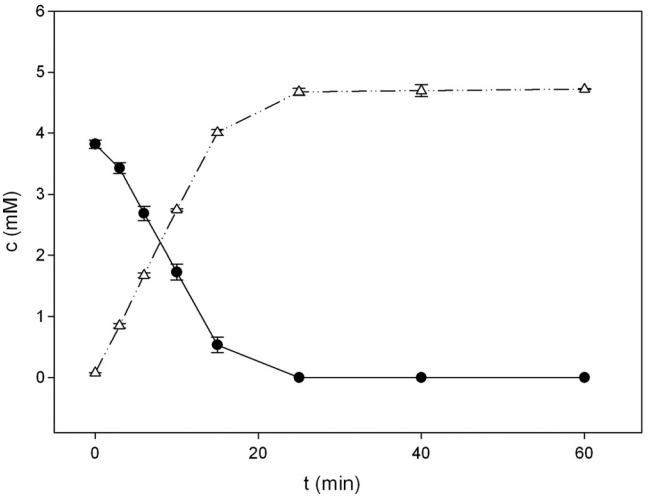


FIG 4 Conversion of 2-methyl-2-phenylpropionitrile to 2-methyl-2-phenylpropionic acid by resting cells of *E. coli* JM109(pIK9) expressing the nitrilase from *P. fluorescens* EBC191. The reaction mixtures (1 ml) contained initially 5 mM 2-methyl-2-phenylpropionitrile (from a 50 mM stock solution in methanol) in 50 mM Na/K-phosphate buffer (pH 7) and resting cells according to an OD_{600} of 15. The reaction mixtures were incubated at 30°C at 1,100 rpm in a thermomixer. The concentrations (c) of 2-methyl-2-phenylpropionitrile (●) and 2-methyl-2-phenylpropionic acid (△) were determined by HPLC.

TABLE 1 Comparison of reaction rates at different pH values of different recombinant derivatives of *E. coli* JM109 heterologously expressing nitrilases from different sources[a]

Plasmid	Source of nitrilase	Assay substrate	pH	Specific activity (U/mg protein)	Relative activity (%)
pIK9	*P. fluorescens* EBC191	2-PPN	7	1.9	100
			5	0.55	28.9
pIK7	*Alcaligenes faecalis*	2-PPN	7	0.4	100
			5	0.007	1.8
pSB520	*Rhodococcus* sp.	Benzonitrile	7	14.1	100
			5	0.002	0.01
pDHE22	*Synechocystis* sp.	Benzonitrile	7	0.1	100
			5	0.006	6

[a] The reactions were performed with resting cells in 50 mM Na/K-phosphate buffer (pH 7) or 100 mM Na-citrate buffer (pH 5). The initial substrate concentrations were 5 mM. 2-PPN, 2-phenylpropionitrile.

incubated at pH 7 and pH 5 with stable nitriles as substrates, and the reaction rates were compared. These experiments demonstrated that resting cells which expressed the nitrilase from *P. fluorescens* showed the highest residual activity at pH 5 (Table 1). A pronounced activity of the nitrilase from *P. fluorescens* at acidic pH values was observed during previous experiments with the purified enzyme (20).

Conversion of acetophenone cyanohydrin by the nitrilase from *P. fluorescens* EBC191. The experiments described above demonstrated that cells of *E. coli* JM109(pIK9) were able to convert sterically hindered phenylacetonitriles at the pH value required for the enantioconservative hydrolysis of acetophenone cyanohydrin. In addition, it was previously shown that the nitrilase activity in intact cells was more acid tolerant than that in cell extracts (38). Therefore, resting cells of *E. coli* JM109(pIK9) were incubated with acetophenone cyanohydrin in Na-citrate buffer at pH 4.5, and it was found that the whole-cell catalyst indeed converted the hydroxynitrile to the corresponding acid (atrolactate) and amide (atrolactamide) at a ratio of about 3.4:1 (Fig. 5A).

The enantiomeric composition of the products formed from acetophenone cyanohydrin was analyzed. Thus, it was found that the nitrilase preferentially formed the (*R*)-form of the acid, and an enantiomeric excess (*ee*) of 51% was calculated when about 30% of the substrate was converted. The *ee* value of the amide could not be quantified because the signal of the (*R*)-amide coeluted with the citrate used as a buffering reagent and some undefined compounds excreted by the resting cells. Nevertheless, from the quantification of the amounts of (*R*,*S*)-atrolactamide determined by achiral HPLC and (*S*)-atrolactamide (quantified by chiral HPLC), it could be concluded that more than 90% of the formed amide consisted of the (*S*)-amide (Fig. 5B).

Generation of a model of the nitrilase by homology modeling. The analysis of the substrate specificity of the nitrilase from *P. fluorescens* EBC191 described above demonstrated that 2-methyl-2-phenylpropionitrile was converted with significantly lower conversion rates than 2-phenylpropionitrile (subsequently, it was found that acetophenone cyanohydrin was also hydrolyzed much more slowly than its nonmethylated counterpart, mandelonitrile [see below]). This indicated that the reaction rates were limited by steric effects due to the additional substituents at the carbon atom adjacent to the nitrile group. This could be rationalized because the carbon atom of the nitrile group forms a covalent bond to the

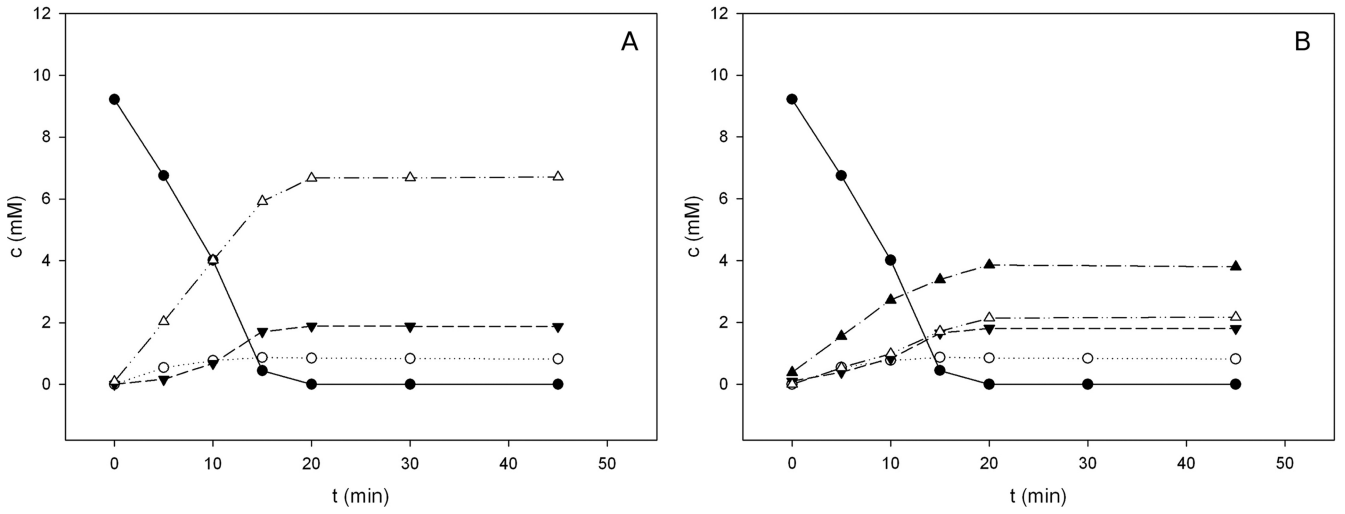


FIG 5 Conversion of acetophenone cyanohydrin by *E. coli* JM109(pIK9). Resting cells (OD_{600} = 42) were incubated in Na-citrate buffer (100 mM; pH 4.5) with 10 mM acetophenone cyanohydrin at 30°C. (A) The reaction was analyzed by achiral HPLC, and the concentrations (c) of acetophenone cyanohydrin (●), atrolactamide (▼), atrolactate (△), and acetophenone (○) were determined. (B) Chiral HPLC was applied in order to quantify the formation of (*S*)-atrolactate (△), (*R*)-atrolactate (▲), and (*S*)-atrolactamide (▼). (The concentrations of acetophenone cyanohydrin [●] and acetophenone [○] were determined as described in panel A.)

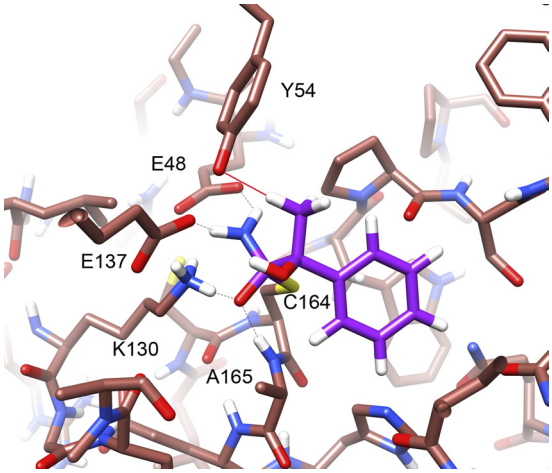


FIG 6 Model of the catalytic center of the nitrilase from *P. fluorescens* EBC191 with a covalently bound tetrahedral intermediate of (*S*)-acetophenone cyanohydrin (purple). The model of the nitrilase (brown) was derived from the structures of the aliphatic amidase from *Nesterenkonia* sp. (PDB accession number 3HKX) and the *D. melanogaster* beta-alanine synthase D subunit (accession number 2VHI). The relative locations of E48, Y54, K130, E137, C164, and the backbone at position 165 are widely conserved. In the model, their locations were derived from those reported under accession number 3HKX. The location of the methyl group of (*S*)-acetophenone cyanohydrin is constrained by the orientation of the tetrahedral intermediate, as explained in the text. In this orientation, there is a clash with the side chain of Tyr54, indicated by the red line.

catalytically active cysteine residue of the enzyme, leading first to a thioimidate intermediate (42) and then to a tetrahedral intermediate from which either the ammonia leaves to form the thioester and, ultimately, the acid product or the cysteine leaves and the amide product is formed (23, 51). The largest number of known constraints on modeling the intermediates and, hence, locating the substrate is at the tetrahedral intermediate. At this time, the nitrile carbon is covalently bound to the cysteine (C164), and the oxyanion is hydrogen bonded to both the catalytic lysine (K130) and the backbone amide of Ala165 (1, 6). We postulate here that the amino group is located between the two conserved active-site carboxyls arising from Glu48 and Glu137. The positioning of the surrounding, nonconserved, active-site residues was accomplished by homology with the amidase from *Nesterenkonia* sp. (PDB accession number 3HKX) (27) and the D subunit of the beta-alanine synthase from *D. melanogaster* (accession number 2VHI) (33). The model suggests that the tyrosine residue at position 54 is involved in a steric clash with one of the groups attached to the asymmetric α-carbon in sterically demanding nitriles (Fig. 6).

Analysis of the nitrilase mutant Tyr54Ala. In order to estimate the influence of the size of the amino acid residue at position 54 on the conversion of sterically demanding substrates, a Tyr54Ala mutant was generated by site-directed mutagenesis and analyzed with respect to the relative activities with acetophenone cyanohydrin and mandelonitrile, amide formation, and the enantioselectivity of the reactions. The mutant converted acetophenone cyanohydrin with about the same relative activity (compared to mandelonitrile) as that of the wild type but surprisingly formed significantly reduced amounts of amides from mandelonitrile and acetophenone cyanohydrin. The enzyme variant differed strikingly from the wild-type enzyme in the kinetics of acetophenone cyanohydrin conversion and product formation. This already became evident during the achiral analysis of the turnover experiments in the form of a pronounced acceleration of substrate disappearance and atrolactate formation when about half of the acetophenone cyanohydrin was converted (Fig. 7A). Reaction kinetics of this type were not observed with the wild-type enzyme (Fig. 5A). The chiral analysis of the reaction demonstrated that this effect was due to the fact that the Tyr54Ala variant initially preferentially formed (*R*)-atrolactate and that the acceleration of the reaction rates correlated with the increased level of formation

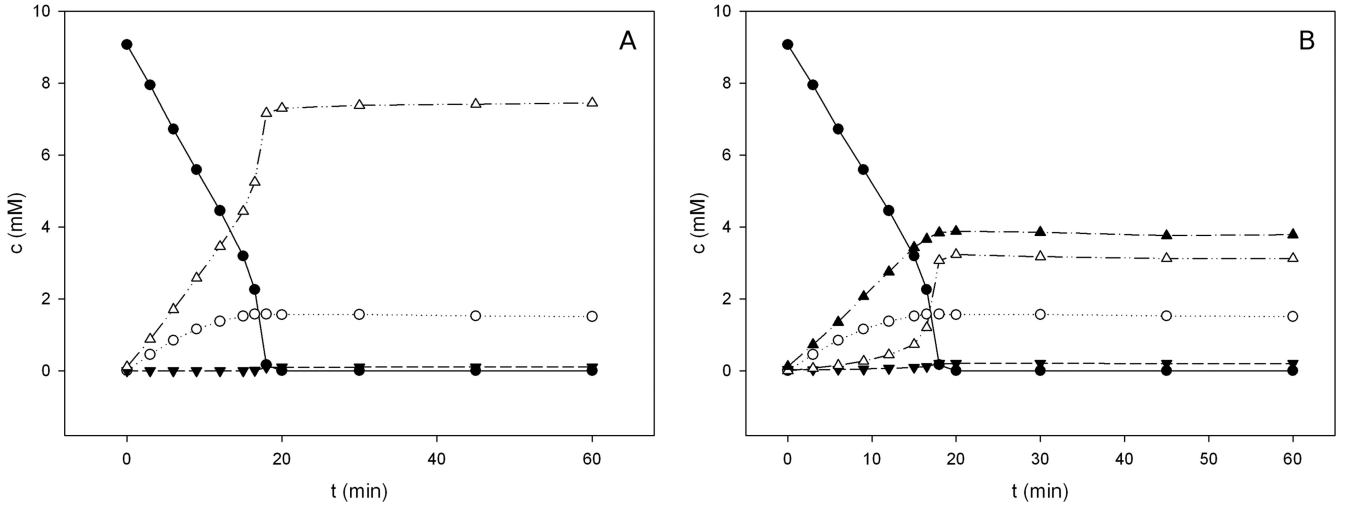


FIG 7 Conversion of acetophenone cyanohydrin by the Tyr54Ala mutant variant of the nitrilase from *P. fluorescens* EBC191. The reaction mixture contained acetophenone cyanohydrin (10 mM) and cells of the respective recombinant *E. coli* strain (OD_{600} = 56) in Na-citrate buffer (100 mM; pH 4.5). The reactions were carried out in a thermomixer at 1,100 rpm at 30°C. (A) The reaction was analyzed by achiral HPLC, and the concentrations (c) of acetophenone cyanohydrin (●), atrolactamide (▼), atrolactate (△), and acetophenone (○) were determined. (B) Chiral HPLC was applied in order to quantify the formation of (*S*)-atrolactate (△), (*R*)-atrolactate (▲), and (*S*)-atrolactamide (▼). (The concentrations of acetophenone cyanohydrin [●] and acetophenone [○] were determined as described in panel A.)

of (*S*)-atrolactate (Fig. 7B). A similar difference between the mutant variant and the wild-type enzyme was not observed with mandelonitrile as the substrate, with which both enantiomers of the acid were formed at almost constant rates throughout the whole biotransformation reaction.

Saturation mutagenesis at Tyr54 of the nitrilase. In order to further analyze the importance of the amino acid residue at position 54 for substrate and reaction specificity, the nitrilase gene was modified in the codon for amino acid residue 54 by using degenerated oligonucleotide primers (see Materials and Methods). From this process, 115 clones were obtained, and 94 of those clones were arbitrarily chosen. The bacterial cultures were induced with rhamnose and tested for the conversion of acetophenone cyanohydrin at pH 4.5. Three clones that converted acetophenone cyanohydrin with about twice the activity of that of the wild type were identified. The genes were sequenced, and it was found that all of them carried the mutation Tyr54Met.

In addition, several clones that formed significantly decreased relative amounts of atrolactamide from acetophenone cyanohydrin (<5% in relationship to the atrolactate formed) were identified. The relevant genes were sequenced, and it was found that these variants carried the mutations Tyr54Leu, Tyr54His, Tyr54Pro, Tyr54Cys, and Tyr54Val.

A third set of mutants, with Tyr54Lys, Tyr54Arg, andTyr54Ile mutations, no longer converted acetophenone cyanohydrin and were also inactive with mandelonitrile or 2-phenylpropionitrile.

Conversion of different sterically demanding nitriles by the enzyme variants. The conversion of acetophenone cyanohydrin, mandelonitrile, 2-phenylpropionitrile, and 2-methyl-2-phenylpropionitrile by the Tyr54Ala, Tyr54Met, Tyr54Val, and Tyr54Pro enzyme variants was subsequently analyzed in detail. In addition, the Tyr54Phe enzyme variant was also examined in order to probe the effect of the phenolic group. Thus, it was found that for the Tyr54Met and Tyr54Val mutants, the increased relative activities for the conversion of acetophenone cyanohydrin (versus the rates for the conversion of mandelonitrile) were also reflected by the increased relative activities with 2-methyl-2-phenylpropionitrile (in comparison to the rates with 2-phenylpropionitrile) (Table 2). Furthermore, it was found that the five tested enzyme variants produced with all tested nitriles reduced relative amounts of amides (Table 2). Analysis of the reactions by chiral HPLC showed that the Tyr54Val and Tyr54Pro enzyme variants demonstrated an inhibition of the conversion of (*S*)-acetophenone cyanohydrin in the presence of (*R*)-acetophenone cyanohydrin similar to that observed previously with the Tyr54Ala variant.

Synthesis of atrolactate and atrolactamide from acetophenone plus cyanide by using whole-cell catalysts simultaneously expressing the oxynitrilase from cassava and the nitrilase from *P. fluorescens* EBC191. The experiments described above demonstrated that the nitrilase from *P. fluorescens* EBC191 was able to convert acetophenone cyanohydrin. Furthermore, it was demonstrated previously that the hydroxynitrile lyase from cassava (*M. esculenta*) converted various ketones to the corresponding cyanohydrins (5, 7). Therefore, a previously constructed "bienzymatic catalyst" [*E. coli* JM109(pIK9)(pJOE5361.1)], which simultaneously expressed these two enzymes and which had been successfully used for the production of (*S*)-mandelic acid plus (*S*)-mandeloamide (40), was tested for the conversion of acetophenone plus cyanide to atrolactate. Resting cells of *E. coli*

TABLE 2 Conversion of mandelonitrile, acetophenone cyanohydrin, 2-phenylpropionitrile, or 2-methyl-2-phenylpropionitrile by different nitrilase variants[a]

Nitrilase variant	Relative activity for formation of mandelic acid (%)	Mean *ee* of mandelic acid (%) ± SD	Mean mandeloamide formed (%) ± SD[b]	Mean relative activity for formation of atrolactic acid (%) ± SD	Mean *ee* of atrolactic acid (%) ± SD	Mean atrolactamide formed (%) ± SD[c]	Relative activity for formation of 2-PPA (%)	Mean *ee* of 2-PPA (%) ± SD	Mean 2-PPAA formed (%) ± SD[d]	Mean relative activity for formation of MMA (%) ± SD
WT	100	25 ± 1.7 (*R*)	19 ± 0.5	3.8 ± 0.06	51 ± 2.4 (*R*)	23 ± 1.8	100	62 ± 1.4 (*S*)	0.2 ± 0.01	7.5 ± 0.08
Y54A	100	11 ± 3 (*R*)	0	3.8 ± 0.3	71 ± 3 (*R*)	2 ± 0.5	ND	ND	ND	ND
Y54V	100	10 (*R*)	1	8	69 (*R*)	0.8	100	47 (*S*)	0	18
Y54M	100	27 ± 3 (*R*)	8 ± 0.7	8.5 ± 1.4	86 ± 0.5 (*R*)	7 ± 1.2	100	60 (*S*)	0	24
Y54F	100	27 (*R*)	1.8	3.6	93 (*R*)	16	100	81 (*S*)	0	6
Y54P	100	13 ± 2 (*R*)	0.6 ± 0.02	4.5 ± 0.1	71 ± 0.4 (*R*)	1.5 ± 0.4	ND	ND	ND	ND

[a] The cells of the recombinant *E. coli* strains were grown and harvested as described in Materials and Methods. Resting cells were incubated in 100 mM Na-citrate buffer (pH 4.5) in a thermomixer at 30°C at 1,100 rpm, and the reactions were started by the addition of 10 mM (*R,S*)-mandelonitrile or (*R,S*)-acetophenone cyanohydrin to the mixture. The relative activities were correlated to the activities of the respective mutants for the formation of mandelic acid (equaling 100%). The specific activities at pH 4.5 of the wild-type (WT) strain and the variants expressing the mutant enzymes were calculated from the cell densities applied (OD_{600} of 1, corresponding to 0.25 g cell dry weight/liter). The values for the wild type and Tyr54Ala, Tyr54Val, Tyr54Met, Tyr54Phe, and Tyr54Pro variants were 3.4 ± 0.4, 2.1 ± 0.5, 1.5, 2.3 ± 0.1, 4.2, and 2.2 ± 0.1 U/mg of protein, respectively. In a second set of experiments, resting cells were incubated in 50 mM Na/K-phosphate buffer (pH 7) in a thermomixer at 30°C at 1,100 rpm, and the reactions were started by the addition of 5 mM (*R,S*)-2-PPN or (*R,S*)-2-methyl-2-phenylpropionitrile to the mixture. The relative activities were correlated to the activities of the respective mutants for the formation of 2-PPN (equal to 100%). The specific activities at pH 7 of the wild-type strain and the variants expressing the Tyr54Val, Tyr54Met, and Tyr54Phe mutant enzymes were 2.3 ± 0.04, 0.33, 0.31, and 0.32 U/mg of protein, respectively. 2-PPA, 2-phenylpropionic acid; 2-PPAA, 2-phenylpropionamide; MMA, 2-methyl-2-phenylpropionic acid; ND, not determined.

[b] Mandeloamide/(mandeloamide + mandelic acid).

[c] Atrolactamide/(atrolactamide + atrolactic acid).

[d] 2-Phenylpropionamide/(2-phenylpropionamide + 2-phenylpropionic acid).

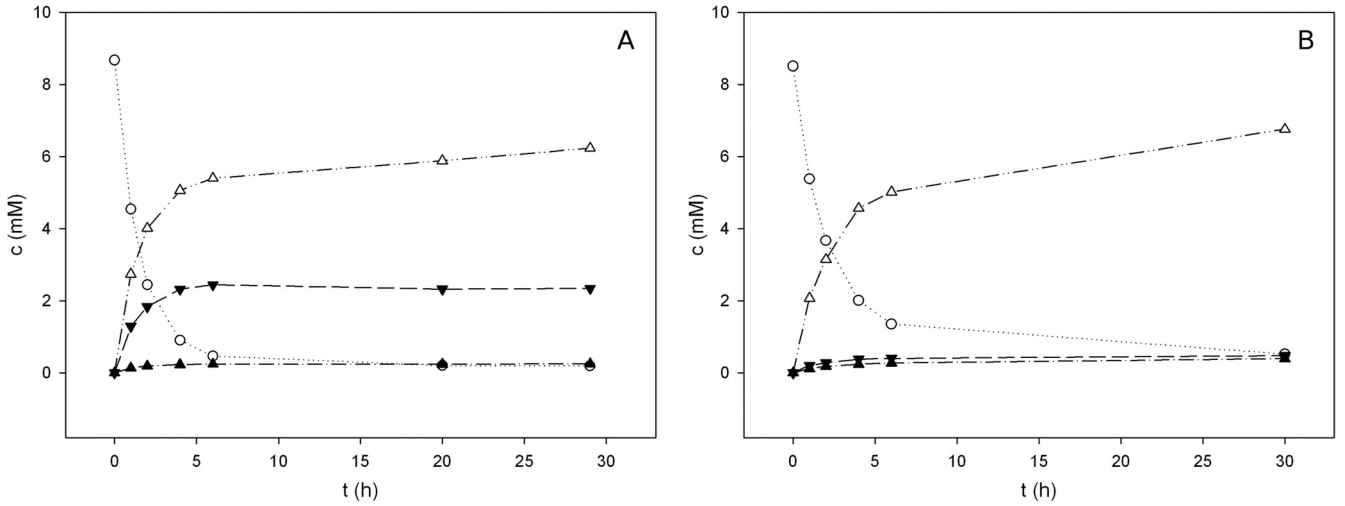


FIG 8 Conversion of acetophenone (10 mM) plus cyanide (30 mM) in Na-citrate buffer (100 mM; pH 4.5) at 30°C by *E. coli* JM109(pIK9)(pJOE5361.1) (A) or *E. coli* JM109(pIK9/Y54V)(pJOE5361.1) (B). The concentrations of (*S*)-atrolactate (△), (*S*)-atrolactamide (▼), (*R*)-atrolactate (▲), and acetophenone (○) were quantified by chiral HPLC.

JM109(pIK9)(pJOE5361.1) converted acetophenone at pH 4.5 to atrolactate and atrolactamide. The whole-cell catalyst formed the (*S*)-enantiomers of atrolactate (*ee* = 92%) and atrolactamide with large enantiomeric excesses (Fig. 8A). This was in sharp contrast to the situation when acetophenone cyanohydrin was converted by the whole-cell catalyst that expressed only nitrilase activity, because in this case, the (*R*)-enantiomer of atrolactate was formed preferentially (Fig. 5B). Therefore, this result confirmed the functionality of the oxynitrilase in the reaction system.

The bienzymatic catalyst, which expressed the wild-type nitrilase from *P. fluorescens* EBC191, converted acetophenone plus cyanide to atrolactate and atrolactamide at a ratio of about 2.5:1 (Fig. 8A). In order to improve the degree of acid formation, a second bienzymatic catalyst was constructed, in which the wild-type nitrilase was replaced by the Tyr54Val nitrilase variant, which demonstrated a decreased level of formation of atrolactamide (Table 2). Thus, a bienzymatic catalyst which formed (*S*)-atrolactate with an *ee* value of 89% and only 4% atrolactamide was obtained (Fig. 8B).

DISCUSSION

Nitrilases are traditionally grouped according to their substrate preferences as aliphatic nitrilases, aromatic nitrilases (respectively "benzonitrilases"), or arylacetonitrilases (32). The first synthetic applications for nitrilases have been established for aromatic nitrilases (especially by using the enzyme from *Rhodococcus rhodochrous* J1), and examples for the conversion of compounds such as 3-cyanopyridine to nicotinic acid or cyanopyrazine to pyrazinoic acid have been investigated (24, 30). Subsequently, several biotransformation reactions which utilize aliphatic nitrilases or arylacetonitrilases were also described (35, 52, 53). Arylacetonitrilases are able to convert α-substituted phenylacetonitriles and are therefore interesting for the enantioselective synthesis of various α-substituted carboxylic acids (and amides). This type of reaction has already been industrialized for the production of (substituted) (*R*)-mandelic acid(s) from racemic mandelonitrile(s) (26). The results obtained during the present study suggest that the synthetic potential of arylacetonitrilases can be even further extended to the conversion of nitriles that contain a quaternary carbon atom adjacent to the nitrile group (as, e.g., present in 2-methyl-2-phenylpropionitrile) or sterically similar demanding substrates, such as acetophenone cyanohydrin. This is, to the best of our knowledge, the first report showing that nitrilases are able to convert these types of compounds. There are also only very few examples in the literature that nitrile hydratases are able to convert nitriles that harbor a quaternary carbon atom in the α-position to the nitrile group. Thus, it was demonstrated that nitrile hydratases convert 2,2′-azobis
(2-methylpropionitrile) and 1-benzyl-2-methylazetidine-2-carbonitriles to the corresponding amides (11, 25).

The nitrilase from *P. fluorescens* EBC191 not only converts a broad range of sterically demanding nitriles but also is more active under acidic conditions than other nitrilases. This was initially indicated by a comparison of the pH optimum curve obtained by Kiziak et al. (20) for the purified nitrilase from *P. fluorescens* EBC191 with the corresponding data for various other nitrilases collected previously by Banerjee et al. (2). These observations were further substantiated by whole-cell experiments with recombinant *E. coli* strains synthesizing different nitrilases that were performed in the course of the present study. The combination of the ability to convert sterically demanding nitriles and to function under rather acidic conditions qualified the nitrilase from *P. fluorescens* EBC191 for the conversion of acetophenone cyanohydrin and also for the construction of "bienzymatic catalysts" in combination with hydroxynitrile lyases. The activity of the (*S*)-oxynitrilase in the bienzymatic catalyst was clearly demonstrated, as the relevant clones preferentially synthesized (*S*)-atrolactate. This was in contrast to the acetophenone cyanohydrin-converting clones, which harbored only the nitrilase activity and preferentially produced (*R*)-atrolactate. These experiments also demonstrated another advantage of the bienzymatic system, because the resting cells converted acetophenone almost quantitatively to the products. This was an interesting observation, as it was repeatedly observed previously that (*S*)-specific oxynitrilases do not quanti-

tatively convert aromatic ketones to the hydroxynitriles, presumably because the equilibrium of these reactions is on the side of the oxo compounds (5, 7, 15, 49). This problem was solved in our system, as the nitrilase activity removed the hydroxynitrile from the reaction mixture.

Molecular analyses of the substrate and reaction specificities of nitrilases are still severely hampered by the absence of an experimentally determined crystal structure. Nevertheless, during the last years, the first examples of site-directed mutagenesis experiments have been described. Thus, Yeom and coworkers generated a model of the active site of the benzonitrilase from *Rhodococcus rhodochrous* ATCC 33278 and verified this model by the generation of mutants with modified activities for aliphatic nitriles or *meta*-substituted benzonitriles (54, 55). Our group has recently demonstrated for the nitrilase from *P. fluorescens* EBC191 that large amino acid residues in the direct neighborhood of the catalytic active cysteine residue are a prerequisite for the preferred formation of (*R*)-mandelic acid from (*R*,*S*)-mandelonitrile (22, 41).

The Tyr54Ala enzyme variant that was produced in the course of the present study was originally generated because it was expected that the tyrosine residue at position 54 might cause some steric repulsion of large substrates. Multiple-sequence alignments demonstrated that the corresponding tyrosine residue is highly conserved among different nitrilases and amidases from bacterial, plant, or fungal sources (21, 45). The importance of this residue for the catalytic activity of nitrilases was experimentally verified during our studies, as several of the enzyme variants generated (e.g., Tyr54Ile, Tyr54Lys, and Tyr54Arg) were completely inactive for the conversion of acetophenone cyanohydrin, mandelonitrile, and 2-phenylpropionitrile. The importance of the respective tyrosine residues for the nitrilase reaction was also confirmed by enzyme variants such as the Tyr54Ala, Tyr54Val, and Tyr54Pro variants, which demonstrated significantly reduced degrees of amide formation from acetophenone cyanohydrin and mandelonitrile. A general reduction in the degree of amide formation was observed previously for other variants of the nitrilase from *P. fluorescens* EBC191. Thus, an exchange of the cysteine residue directly adjacent to the catalytically active cysteine residue (toward the amino terminus) by an alanine (Cys163Ala) or serine (Cys163Ser) residue or the introduction of a sterically demanding amino acid residue "on the other side" of the catalytically active cysteine residue (e.g., in Ala165Phe) resulted in a significant suppression of the nitrile hydratase activity of the nitrilase (22, 41). The similar effects caused by mutations such as Cys163Ala, Ala165Phe, or Tyr54Ala on the reduction of amide formation in combination with the observed deleterious effect of other mutations at position 54 suggested that the amino acid residue Tyr54 is indeed located close to the catalytic center and thus indicate the correctness of the structural enzyme model.

The kinetics of the turnover of acetophenone cyanohydrin significantly differed between the wild-type nitrilase and several of the mutants that were modified at position 54. This was especially evident for the Tyr54Ala enzyme variant. For this variant, it was found that the formation of (*S*)-atrolactate accelerated as soon as about half of the (*R*,*S*)-acetophenone cyanohydrin was converted. In the first half of this reaction, mostly (*R*)-atrolactate was formed, and thus, the (*R*)-acetophenone cyanohydrin was depleted. This finding suggested that the increase in the rate of conversion of (*S*)-acetophenone cyanohydrin correlated with the disappearance of (*R*)-acetophenone cyanohydrin. Thus, the peculiar situation occurred that the rate of the conversion of the nonpreferred (*S*)-enantiomer was higher than the conversion rate of the (preferred) (*R*)-enantiomer in the Tyr54Ala variant. This indicated that the covalent intermediate of the (*S*)-enantiomer was hydrolyzed with a higher rate than that of the respective intermediate of the (*R*)-enantiomer. Similar effects of a mutation on the chiral discrimination of an enzyme were recently described for an epoxide hydrolase from *Agrobacterium radiobacter* (46). A pronounced suppression of the turnover of (*S*)-acetophenone cyanohydrin by the presence of (*R*)-acetophenone cyanohydrin was evident for the Tyr54Ala, Tyr54Val, and Tyr54Pro mutants. This might indicate that the small size and/or apolar character of the alanine, valine, or proline residues could cause the observed decrease in the affinity of the enzyme variants for (*S*)-acetophenone cyanohydrin.

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