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ORIGINAL PAPER

Conversion of aliphatic nitriles by the arylacetonitrilase from *Pseudomonas fluorescens* EBC191

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Abstract

The conversion of aliphatic nitriles by the arylacetonitrilase from *Pseudomonas fluorescens* EBC191 (NitA) was analyzed. The nitrilase hydrolysed a wide range of aliphatic mono- and dinitriles and showed a preference for unsaturated aliphatic substrates containing 5–6 carbon atoms. In addition, increased reaction rates were also found for aliphatic nitriles carrying electron withdrawing substituents (e.g. chloro- or hydroxy-groups) close to the nitrile group. Aliphatic dinitriles were attacked only at one of the nitrile groups and with most of the tested dinitriles the monocarboxylates were detected as major products. In contrast, fumarodinitrile was converted to the monocarboxylate and the monocarboxamide in a ratio of about 65:35. Significantly different relative amounts of the two products were observed with two nitrilase variants with altered reaction specifities. NitA converted some aliphatic substrates with higher rates than 2-phenylpropionitrile, which is one of the standard substrates for arylacetonitrilases. This indicated that the traditional classification of nitrilases as "arylacetonitrilases", "aromatic" or "aliphatic" nitrilases might require some corrections. This was also suggested by the construction of some variants of NitA which were modified in an amino acid residue which was previously suggested to be essential for the conversion of aliphatic substrates by a homologous nitrilase.

Keywords Biotransformation · Nitrilase · Regioselective hydrolysis · Substrate specifity

Introduction

Nitriles with the general formula R-CN are produced in nature by several groups of organisms, e.g. plants, fungi, insects and millipedes. They function in biology often as repellents against predators, but also for the detoxification of cyanide and as intermediates in (secondary) metabolism (Caspar and Spiteller 2015; Dadashipour et al. 2015; Legras et al. 1990; O'Reilly and; Turner 2003; Zagrobelny et al. 2004). Nitriles are also important products and intermediates in the chemical industry. As nitriles are found as natural and man-made products, it is not surprising that several organisms produce nitrile converting enzymes. The most important groups of nitriles converting enzymes are nitrile hydratases which convert nitriles to amides and nitrilases which hydrolyse nitriles directly to carboxylic acids and ammonia (Banerjee et al. 2002; Brady et al. 2004; DiCosimo 2002; Prasad and Bhalla 2010; Gong et al. 2017).

Nitrilases are found in plants, fungi, and bacteria. They are oligomeric proteins which usually form peculiar spiral shaped structures that can be visualized by electron microscopy (Brenner 2002; Thuku et al. 2008). The catalytic mechanism involves an intermediately formed covalent bond between the carbon-atom of the nitrile group and a cysteine residue of the enzyme. In addition, lysine and glutamate residues take part in the enzymatic reaction (Fernandes et al. 2006; Kobayashi et al. 1992a; Sosedov and Stolz 2015; Stevenson et al. 1992). During the last years, the efforts of several scientific groups have shown that nitrilases convert an almost countless number of nitriles (Banerjee et al. 2002; DiCosimo 2002; Martinková and Mylerová 2003; Martinková and Křen 2010; Singh et al. 2006; Wang 2005). There exist several established and potential applications for these enzymes in biotechnology. Thus, enantioselective and/or regioselective nitrilase reactions can be used for the synthesis of high-value carboxylic acids from nitriles (Gong et al. 2017; Martinková and Křen 2010; Martinková and Mylerová 2003; Singh et al. 2006).

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Nitrilases are generally grouped according to their substrate preferences as arylacetonitrilases, or aromatic or aliphatic nitrilases (Harper 1977a, b; Kobayashi et al. 1990a, b; Nagasawa et al. 1990). An arylacetonitrilase from *Pseudomonas fluorescens* EBC191 has been studied for several years in our laboratory in respect to substrate and reaction specifity, enantioselectivity, and structure-activity-relationships (Baum et al. 2012; Fernandes et al. 2006; Kiziak et al. 2005, 2007; Kiziak and Stolz 2009; Sosedov et al. 2010; Sosedov and Stolz 2014, 2015). In the course of these investigations it was found that the enzyme converted in addition to several arylacetonitriles also some aliphatic nitriles (Heinemann et al. 2003b; Kiziak et al. 2005). This suggested that the traditional separation between aliphatic nitrilases and arylacetonitrilases might be somehow artificial. Therefore, in the present study the conversion of aliphatic substrates by the arylacetonitrilase from *P. fluorescens* EBC191 was studied in greater detail in order to systematically analyze the differences between aliphatic and arylacetonitrilases. Furthermore, it was attempted to analyze if the conversion of aliphatic nitriles by arylacetonitrilases might be useful for synthetic purposes.

Materials and methods

Bacterial strains and plasmids

The construction of plasmid pIK9 has been described before. The plasmid encodes the gene for the nitrilase from *Pseudomonas fluorescens* EBC191 under the control of a rhamnose-inducible promoter (Kiziak et al. 2005).

Escherichia coli JM109 was used as host strain for pIK9 and its derivatives.

Preparation of resting cells with nitrilase activity

A preculture of *Escherichia coli* JM109(pIK9) was grown overnight in LB-medium plus 100 µg/ml ampicillin. This preculture (5 ml) was used to inoculate 500 ml of LB-medium plus 100 µg/ml ampicillin plus 0.2% (w/v) L-rhamnose in 3 l Erlenmeyer flasks with baffles. The flasks were shaken for 18 h at 30 °C and 150 rpm. The cells were harvested by centrifugation (10 min, 4 °C, 4600 rpm), washed in cold Tris/HCl buffer (25 mM, pH 7.5) and resuspended in Tris/HCl buffer (25 mM, pH 7.5).

Determination of the nitrilase activities by quantitation of the released ammonia

The formation of ammonia was determined either in microtiter plates (initial screening) or in a slightly larger scale in Eppendorf tubes. For the microtiter plate assays, the cell suspensions ($OD_{600\ nm} = 0.2–37$) were divided (180 µl each) to the chambers of a 96-well microtiter plate (Cellstar, 400 µl volume/well; Greiner Bio-One, Kremsmünster, Austria). Then, the reactions were started by the addition of 20 µl of a stock solution of the respective nitrile (100 mM in methanol). The microtiter plate was incubated at 30 °C and 600 rpm on a shaker. After different time intervals aliquots (40 µl each) were taken and the amount of ammonia formed immediately quantified or after the removal of the cells in a microtiter plate centrifuge (20 min, 4600 rpm).

The release of ammonia was basically determined as described by Black et al. (2015). The individual reaction chambers of a 96-well microtiter plate (Cellstar, 400 µl volume/well; Greiner Bio-One) contained dimethylsulfoxide (31 µl) and 22 µl of an *ortho*-phthalaldehyde (OPA)-reagent (80 mg OPA in 400 µl methanol dissolved in 40 ml 15 mM Na-tetraborate, pH 9.25). The aliquots taken from the biotransformation experiments were added (11 µl per well) and then 11 µl of 10% (v/v) trichloroacetic acid. Next, dimethylsulfoxide (75 µl) was added, the test solutions intensively mixed and the plates incubated for 10 min at room temperature. Finally, the absorbance at 675 nm was measured in a microtiter plate reader (EON, BioTek, Winooski, VT, USA). Calibration curves were generated by using defined concentrations of NH_4Cl (1–15 mM).

One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmol of ammonia/min. The specific activities of the whole cell catalysts were calculated on the basis of the assumption that an optical density ($OD_{600\ nm}$) of 1 corresponded to 125 mg/l of protein.

Determination of the nitrilase activities by HPLC

The biotransformation of the non-hydroxylated nitriles was performed in Tris/HCl-buffer (25 mM, pH 7.5). The cells were resuspended to optical densities ($OD_{600\ nm}$) of 0.3–130 in the buffer and cell suspensions (0.9 ml each) were transferred to Eppendorf tubes. The tubes were incubated at 30 °C in a thermomixer (600 rpm) and the reactions started by the addition of 100 µl of methanolic stock solutions of the respective nitriles (100–150 mM). The starting concentrations of the nitriles were 5–100 mM. Aliquots (80 µl each) were taken at different time points, the cells removed by centrifugation in an Eppendorf centrifuge (2 min, 14.000 rpm), and the supernatants analysed by HPLC.

The reactions with α-hydroxynitriles were basically performed as described above, but more acidic reaction buffers used [50 mM Na-KH-phthalate- (pH 5 or pH 6) or Na-citrate-buffer (pH 5)] and the reactions stopped by the addition of 1 M HCl (10 µl) prior to the centrifugation step.

HPLC

The turn-over of the aliphatic nitriles was quantified by high pressure liquid chromatography (HPLC) (Agilent 1100) using a 250 mm × 4 mm Lichrospher RP18 column (Trentec Analysentechnik, Rutesheim, Germany). The nitriles and their corresponding amides and acids were either analysed by using solvent systems consisting of 5–30% (v/v) methanol and 0.3% (v/v) H_3PO_4 in H_2O or a solvent system composed of 5 mM KH_2PO_4/H_3PO_4, pH 2 plus acetonitrile, with a usual flow rate of 0.4 ml/min (Table 1).

The solvent system used for the analysis of the turn-over of 2-phenylpropionitrile contained 50% (v/v) methanol and 0.3% (v/v) H_3PO_4 in H_2O.

The separated compounds were detected by using a refractive index detector (Agilent 1260) or an optical detector (Agilent G1315B) at wavelengths of 195 and 210 nm.

Site-directed mutagenesis

The site-directed mutations were generated by using the "QuikChange site directed mutagenesis kit" according to the instructions given by the supplier (Agilent, Santa Barbara, CA). The mutations were verified by DNA sequencing (GATC Biotech, Konstanz, Germany).

Nuclear magnetic resonance (NMR) spectra

The NMR spectra were recorded with a Bruker 400 MHz NMR spectrometer.

Protein modelling

The program Yasara (version 17.4.17) was used for the homology modelling of proteins (Krieger et al. 2002). The structure of the nitrilase from *Synechocystis* sp. PCC6803 (PDB 3WUY) was used as template (Zhang et al. 2014). Valeronitrile was manually docked to the catalytical active

Table 1 HPLC conditions used for the analysis of the nitriles and reaction products

Substance	Solvent system	Detector	Column temperature (°C)	Retention time (min)
Acrylonitrile	63% (v/v) 5 mM KH_2PO_4/H_3PO_4, pH 2.9 + 37% (v/v) acetonitrile	UV/Vis (λ = 190 nm)	7	8.2
Acrylamide	63% (v/v) 5 mM KH_2PO_4/H_3PO_4, pH 2.9 + 37% (v/v) acetonitrile	UV/Vis (λ = 190 nm)	7	6.0
Acrylic acid	63% (v/v) 5 mM KH_2PO_4/H_3PO_4, pH 2.9 + 37% (v/v) acetonitrile	UV/Vis (λ = 190 nm)	7	6.7
Adiponitrile (Hexanedinitrile)	63%(v/v)5 mM KH_2PO_4/H_3PO_4, pH 2.9 + 37% (v/v) acetonitrile	RID	7	8.7
Allylcyanide (3-Butenenitrile)	74.7% (v/v) H_2O, 25% (v/v) methanol, 0.3% (v/v) H_3PO_4	UV/Vis (λ = 210 nm)	20	13.6
Vinylacetic acid (3-Butenoic acid)	74.7% (v/v) H_2O, 25% (v/v) methanol, 0.3% (v/v) H_3PO_4	UV/Vis (λ = 210 nm)	20	9.8
2-Chloropropionitrile	63%(v/v)5 mM KH_2PO_4/H_3PO_4, pH 2.9 + 37% (v/v) acetonitrile	UV/Vis (λ = 210 nm)	5	11.5
2-Chloropropionic acid	63%(v/v)5 mM KH_2PO_4/H_3PO_4, pH 2.9 + 37% (v/v) acetonitrile	UV/Vis (λ = 210 nm)	5	7.3
Fumarodinitrile	94.7% (v/v) H_2O, 5% (v/v) methanol, 0.3% (v/v) H_3PO_4	UV/Vis (λ = 210 nm)	5	11.0
Fumaric acid	94.7% (v/v) H_2O, 5% (v/v) methanol, 0.3% (v/v) H_3PO_4	UV/Vis (λ = 210 nm)	5	13.2
3-Hexenedinitrile	63%(v/v)5 mM KH_2PO_4/H_3PO_4, pH 2.9 + 37% (v/v) acetonitrile	UV/Vis (λ= 190 nm)	7	8.6
2-Hydroxy-3-butenenitrile	94.7% (v/v) H_2O, 5% (v/v) methanol, 0.3% (v/v) H_3PO_4	UV/Vis (λ = 210 nm)	5	10.2
2-Hydroxybutyronitrile	94.7% (v/v) H_2O, 5% (v/v) methanol, 0.3% (v/v) H_3PO_4	RID	5	15.3
Lactonitrile	94.7% (v/v) H_2O, 5% (v/v) methanol, 0.3% (v/v) H_3PO_4	RID	5	8.9
2-Methyleneglutarodinitrile	63%(v/v)5 mM KH_2PO_4/H_3PO_4, pH 2.9 + 37% (v/v) acetonitrile	UV/Vis (λ = 190 nm)	7	8.9
Valeronitrile	63%(v/v)5 mM KH_2PO_4/H_3PO_4, pH 2.9 + 37% (v/v) acetonitrile	RID	5	14.3
Valeric acid	63%(v/v)5 mM KH_2PO_4/H_3PO_4, pH 2.9 + 37% (v/v) acetonitrile	RID	5	9.6

The given average retention times relate to a flow rate of 0.4 ml/min

cysteine residue of NitA and an intermediate constructed, in which the sulphur of the cysteine residue was covalently bound to the C-atom originating from the cyanide carbon atom of the substrate. Subsequently, the amino- and hydroxyl-groups of the resulting quaternary intermediate were manually orientated towards the catalytical active glutamate- (Glu48) and lysine- (Lys130) residues, respectively. Finally, an energy minimization was performed.

Chemicals

The aliphatic nitriles and dinitriles were supplied by Sigma-Aldrich (St. Louis, MO), Lancaster (Ward Hill, MA), Merck (Darmstadt, Germany), Santa Cruz (Dallas, TX) and TCI (Tokyo, Japan).

Results

Screening for aliphatic nitriles which are converted by the nitrilase from *Pseudomonas fluorescens* EBC191

In previously performed studies it was found that the nitrilase from *P. fluorescens* EBC191 (NitA) converted in addition to several arylacetonitriles also 2-acetoxybutenenitrile and valeronitrile (Heinemann et al. 2003b; Kiziak et al. 2005). Therefore, it was tested if NitA could convert a broader range of aliphatic nitriles. Initially, a colorimetric screening was used which allowed to detect the formation of ammonia by a reaction with *ortho*-phthalaldehyde (OPA) to a blue-black chromophore (Black et al. 2015).

Escherichia coli JM109(pIK9) synthesizing NitA was grown in LB-medium (plus ampicillin) and *nitA* induced by the addition of L-rhamnose. Suspensions of resting cells ($OD_{600\ nm} = 0.2–35$) were prepared in Tris/HCl buffer (25 mM, pH 7.5) and incubated in the wells of a deepwell microtiter plate with various saturated and unsaturated aliphatic mononitriles (16.7 mM each) and dinitriles (8.3 mM each) containing 3–19 carbon atoms (Figs. 1, 2). The amount of cells used were optimized for each substrate in order to obtain a clearly detectable coloration within 45 min. The cells were then removed by centrifugation and the samples analyzed by using the color assay. Thus, the release of ammonia was detected from several aliphatic nitriles and significant differences in the intensity of the formed bluish coloration ($\lambda_{max} = 675$ nm) were observed. 2-Chloropropionitrile and valeronitrile were converted among the tested mononitriles with the highest rates, but significant amounts of ammonia were also released from other saturated and unsaturated mononitriles. The release of ammonia was also found with several dinitriles, such as 3-hexenedinitrile, 2-methyleneglutarodinitrile, adiponitrile (hexanedinitrile), and fumarodinitrile. In contrast, no significant release of ammonia was found, when an *E. coli* strain carrying pJOE2775 (= vector control) (Kiziak et al. 2005) was incubated under the same conditions with these nitriles.

Determination of the reaction rates

In order to quantify the reaction rates, resting cells of *E. coli* JM109(pIK9) were incubated with those nitriles which had been identified as putative substrates of NitA and samples taken at different time points. The resting cells released ammonia from most of the nitriles with almost constant rates for at least 6 h (or until the substrates were almost completely converted). The highest reaction rates were observed with 3-hexenedinitrile, 2-chloropropionitrile, and valeronitrile (Table 2). The specific activities of the whole cell catalysts with 3-hexenedinitrile and 2-chloropropionitrile (3.3 and 2.8 U/mg of protein, respectively) appeared higher than those previously found for the conversion of 2-phenylpropionitrile (Kiziak et al. 2007). Therefore, the conversion of 2-chloropropionitrile and 2-phenylpropionitrile were directly compared with the same batch of cells and its was found that 2-chloropropionitrile was indeed converted about 10% faster than 2-phenylpropionitrile.

The comparison of the individual reaction rates suggested that the enzyme preferred unsaturated substrates [see e.g. the conversion of acrylonitrile *vs*. propionitrile, or fumarodinitrile and allylcyanide (3-butenenitrile) vs. butyronitrile, or 3-hexenedinitrile vs. hexanedinitrile (adiponitrile)]. The nitrilase demonstrated the highest activities with aliphatic substrates with chain lengths of 5–6 carbon atoms (e.g. 3-hexenedinitrile or valeronitrile).

The high reaction rates observed with 2-chloropropionitrile correlated well with previously performed experiments with 2-chloro-2-phenylacetonitrile (Fernandes et al. 2006), which also suggested that NitA efficiently converts chlorinated substrates.

The resting cells of *E. coli* JM109(pIK9) were subsequently tested for the conversion of aliphatic α-hydroxynitriles, such as 2-hydroxy-3-butenenitrile, 2-hydroxybutyronitrile, 2-hydroxypropanenitrile (lactonitrile), 2-hydroxyisobutyronitrile (acetone cyanohydrine), and 1,1,1-trifluoroacetone cyanohydrine (Fig. 1). Among this group of substrates, the cells converted 2-hydroxy-3-butenenitrile with the highest activities (0.25 U/mg of protein). For the other tested aliphatic α-hydroxynitriles only specific activities ≤ 0.05 U/mg protein were determined. The comparison of the turn-over rates of the hydroxylated substrates with those observed for the non-hydroxylated substrates suggested that a substitution with a hydroxyl-group

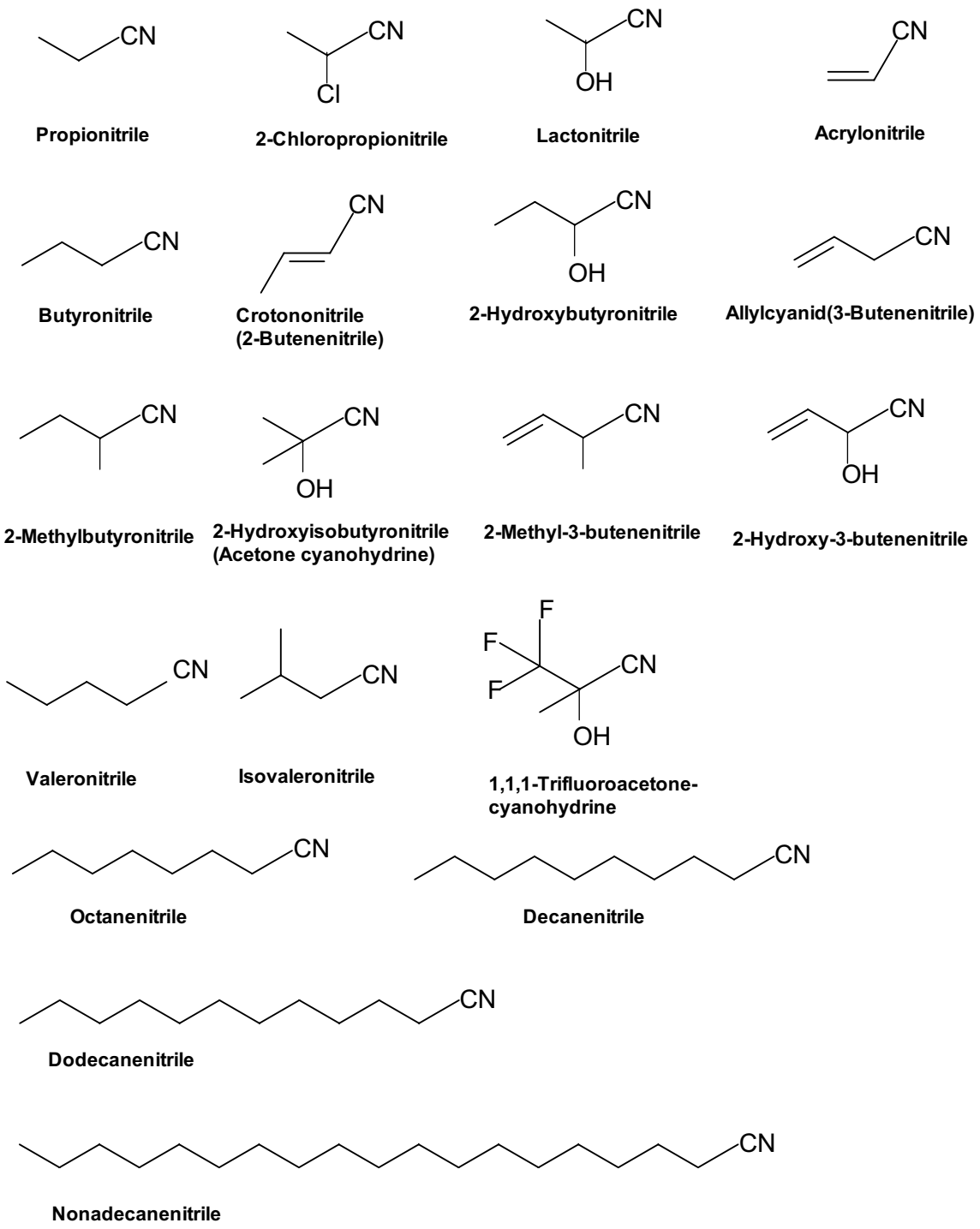

Fig. 1 Aliphatic mononitriles tested in the present study

resulted in slightly increased reaction rates compared to a methyl-substitution (see e.g. the conversion of 2-hydroxy-3-butenenitrile vs. 2-methyl-3-butennitrile or 2-hydroxybutyronitrile vs. 2-methylbutyronitrile) (Table 2).

Stability of aliphatic 2-hydroxynitriles in aqueous solutions

The experiments described above were performed at pH 7.5. 2-Hydroxynitriles decompose under neutral conditions spontaneously to cyanide and aldehydes (or ketones) and

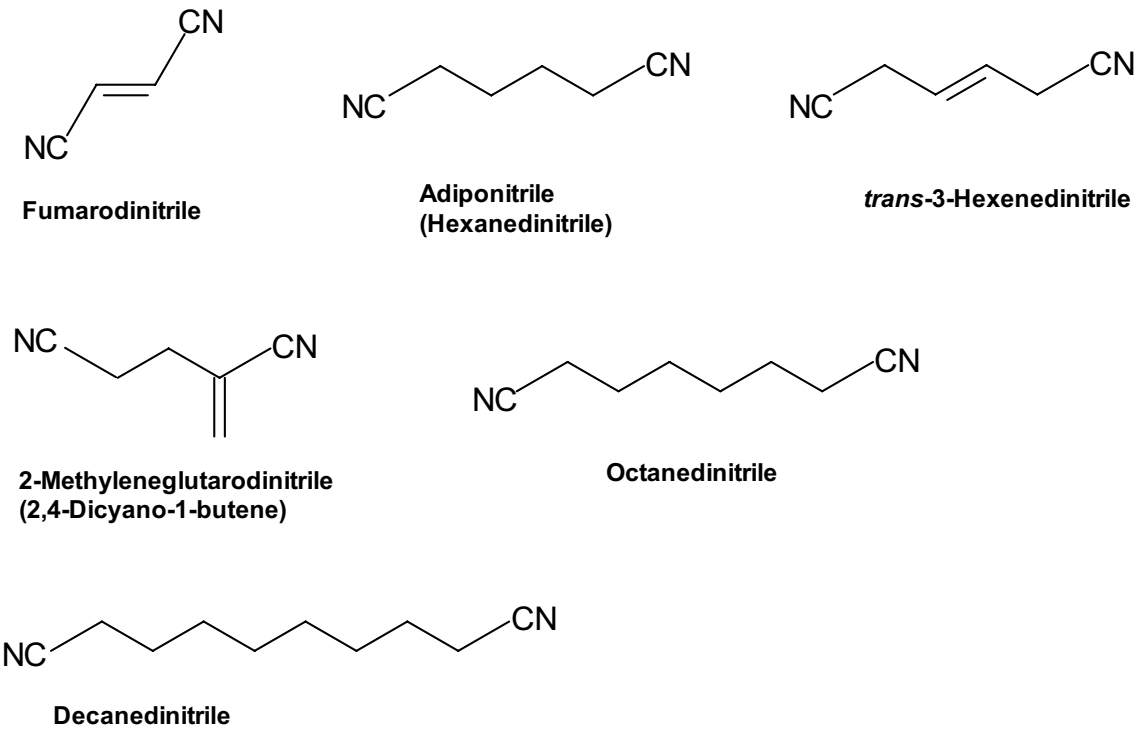

Fig. 2 Aliphatic dinitriles tested in the present study

it is often necessary to perform the enzymatic hydrolysis of α-hydroxynitriles under acidic conditions (Rustler et al. 2007; Sosedov et al. 2009; Yamamoto et al. 1991). Therefore, in order to optimize the reactions, the stability of aliphatic 2-hydroxynitriles was compared at different pH-values and temperatures. Initially, the spontaneous decay of 2-hydroxy-3-butenenitrile to acrolein (Fig. 3) was analysed. These experiments demonstrated that at pH 7.5 and room temperature 2-hydroxy-3-butenenitrile readily decomposed to acrolein (Fig. 4A). This reaction was significantly slowed down by a decrease in the pH (Fig. 4A) and/or a decrease in the incubation temperature to 4–6 °C (Fig. 4B). The comparison of these results with the previously performed analogous experiments with mandelonitrile and acetophenone cyanohydrine (Rustler et al. 2007; Baum et al. 2012) demonstrated that under acidic conditions the stability of the 2-hydroxynitriles tested decreased in the order 2-hydroxy-3-butenenitrile > mandelonitrile > acetophenone cyanohydrin. In subsequent experiments it was demonstrated that lactonitrile and 2-hydroxybutyronitrile were even more stable than 2-hydroxy-3-butenenitrile in aqueous solutions at pH 5 and pH 7.5.

Analysis of the products formed from the mononitriles by HPLC

It was previously shown that the nitrilase from *P. fluorescens* EBC191 converted certain phenylacetonitriles (e.g. mandelonitrile) not only to the carboxylic acids but also formed significant amounts of the corresponding amides (Fernandes et al. 2006; Kiziak et al. 2005). Therefore, HPLC coupled to a diode array (DAD) or a refractive index detector (RID) was used in order to analyze if any amides were formed from 2-chloropropionitrile, valeronitrile, allylcyanide, and acrylonitrile, as these were the four aliphatic mononitriles which were converted according to the ammonia assays with the highest activities. Valeronitrile, allylcyanide, and 2-chloropropionitrile were converted each only to one detectable product, which were identified by comparison with authentic standards as the corresponding acids. The cells converted the substrates to almost equimolar amounts of ammonia and the acids. The conversion of acrylonitrile resulted in a major organic product, which was identified according to its retention time as acrylic acid. Furthermore, traces (≤ 5%) of acrylamide were also detected.

Subsequently, the turn-over of 2-hydroxy-3-butenenitrile was analysed under conditions which sufficiently minimized the spontaneous decay of the substrate. Therefore, resting cells of *E. coli* JM109(pIK9) were incubated with 2-hydroxy-3-butenenitrile at pH 6 and 6 °C. The resting cells converted 2-hydroxy-3-butenenitrile with a specific activity of 0.16 U/mg of protein almost stoichiometrically (< 0.5% of amide formed) to the corresponding carboxylic acid (Fig. 5).

Table 2 Specific activities for the release of ammonia from different aliphatic mono- and dinitriles by resting cells of *E. coli* JM109(pIK9)

Substrate	Specific activity (U/mg)	Relative activity (3-Hexenedinitrile = 100%)
3-Hexenedinitrile	3.25	100
2-Chloropropionitrile	2.79	86
Valeronitrile	0.80	25
Allylcyanid	0.38	12
2-Methyleneglutarodinitrile	0.37	11
Fumarodinitrile	0.36	11
2-Hydroxy-3-butenenitrile	0.25	8
Acrylonitrile	0.15	5
2-Methyl-3-butenenitrile	0.09	3
Adiponitrile	0.07	2
2-Hydroxybutyronitrile	0.05	2
Butyronitrile	0.04	1
Lactonitrile	0.04	1
Isovaleronitrile	0.03	1
Propionitrile	0.03	1
Cis,trans-Crotononitrile	0.02	< 1
Octanedinitrile	0.02	< 1
Decanedinitrile	0.004	< 1
2-Methylbutyronitrile	0.004	< 1

The cells of *E. coli* JM109(pIK9) were cultivated and resting cells prepared in Tris/HCl buffer (25 mM, pH 7.5) as described in the materials and methods section. The resting cells ($OD_{600\ nm}$ = 0.2–36.5) were transferred to Eppendorf tubes (1 ml each). The reactions were started by the addition of the nitriles (10 mM each; from 100 mM methanolic stock solutions). The reaction mixtures were incubated at 23 °C and 600 rpm on a shaker. Aliquots (100 µl each) were taken after different time intervals (0–300 min). The cells were removed by centrifugation (2 min, 12,000 rpm) and the amount of ammonia released determined as described in the materials and methods section

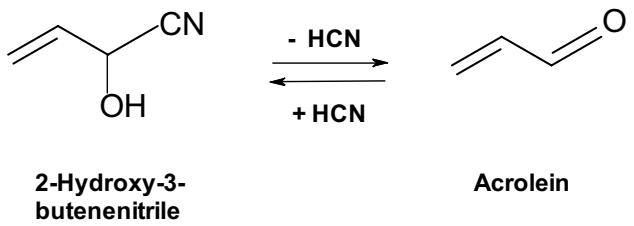

Fig. 3 Spontaneous decay of 2-hydroxybutenenitrile to acrolein

Evidence for the regioselective hydrolysis of aliphatic dinitriles

The experiments summarized in Table 2 demonstrated that NitA converted in addition to aliphatic mononitriles also several aliphatic dinitriles. It has been reported previously that certain nitrilases hydrolyse only one nitrile group of dinitriles and thus generate cyanocarboxylic acids which are of some synthetic value (Bayer et al. 2011; Bengis-Garber and Gutman 1989; Chauhan et al. 2003; Heinemann et al. 2003a; Kobayashi et al. 1990b; Rey et al. 2004; Zhu et al. 2007). In order to test if NitA also shows some regioselectivity for the conversion of aliphatic dinitriles, very high concentrations of *E. coli* JM109(pIK9) ($OD_{600\ nm}$ = 32) were incubated with fumarodinitrile, adiponitrile, 3-hexenedinitrile, 2-methyleneglutarodinitrile, or octanedinitrile (4.2 mM each) and the formation of ammonia measured. Thus, it was found that even after prolonged incubation times (t = 4 h) in none of these experiments more than 4.2 mM of ammonia was formed. This indicated that NitA could only release ammonia from one of the nitrile groups of the tested dinitriles (as the hydrolysis of both nitrile groups of these substrates would result in the formation of 8.4 mM of ammonia, each).

Conversion of fumarodinitrile

The turn-over of the dinitriles was subsequently analysed in more details by HPLC and NMR. Fumarodinitrile (R_t = 11.0 min) was converted to two products (R_t = 10.0 min and R_t = 13.0 min). The R_t-values of both products were different from fumaric acid (R_t = 13.4 min). The signal intensities at λ = 210 nm of the two products indicated that they were formed in a ratio of about 65:35 with a surplus of the product with the longer retention time. Subsequently, the amounts of ammonia formed were determined using the established colorimetric test and it was found that only about 2 mM of ammonia were released after the complete conversion of 5 mM fumarodinitrile (Fig. 6A).

These results indicated that NitA converted fumarodinitrile to a product with a single carboxylic group (= 3-cyanoacrylic acid) and that in addition also a mono- or diamide was formed (Fig. 7). Unfortunately, no commercial standards of these putative products were available. Therefore, fumarodinitrile (10 mM) was converted by resting cells of *E. coli* JM109(pIK9) in K-phosphate buffer (100 mM, pH 7). After the complete conversion of the substrate (detected by HPLC), the cells were removed by centrifugation and the supernatant directly analysed by ^{1}H-NMR. The NMR spectrum showed the presence of two pairs of doublets. The product which was present in a slightly higher concentration gave signals at 6.14, 6.23, 6.77, and 6.83 ppm (J = 16 Hz) and the minor product showed signals at 6.38, 6.42, 6.95, and 6.99 ppm (J = 16 Hz) (Fig. 8).

The presence of two pairs of doublets demonstrated that indeed neither residual amounts of fumarodinitrile nor detectable amounts of fumaric acid were present in the reaction mixture, as these (symmetric) compounds only gave singlets at 6.35 and 6.75 ppm, respectively. (In addition,

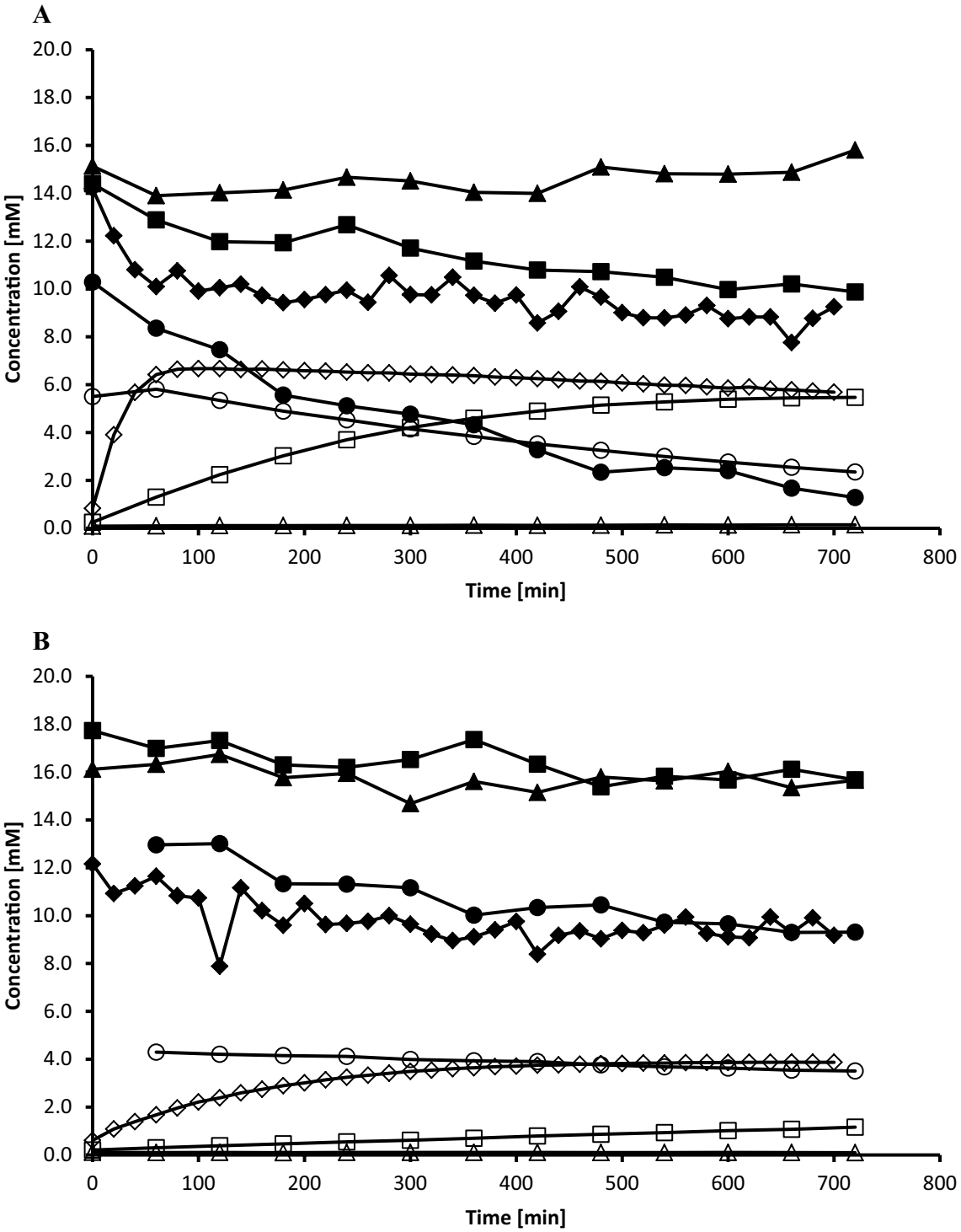

Fig. 4 Stability of 2-hydroxy-3-butenenitrile at 23 °C (**A**) and 5 °C (**B**) at different pH-values. A stock solution of 2-hydroxy-3-butenenitrile (150 mM) was prepared in methanol and diluted in a ratio of 1:9 in K-phosphate (pH 2.9), Na-citrate (pH 5.0, pH 6.0), or Tris/HCl (pH 7.5). The samples were transferred to HPLC sample vessels and analysed using an automatic sample injector. The turn-over of 2-hydroxy-3-butenenitrile (filled symbols) and the formation of acrolein (propenal) (open symbols) were determined by HPLC at pH 2.9 (▲, Δ), pH 5.0 (■, □), pH 6.0 (◆, ◇), and pH 7.5 (●, ○) and the concentrations of the substrate and product presented in mM

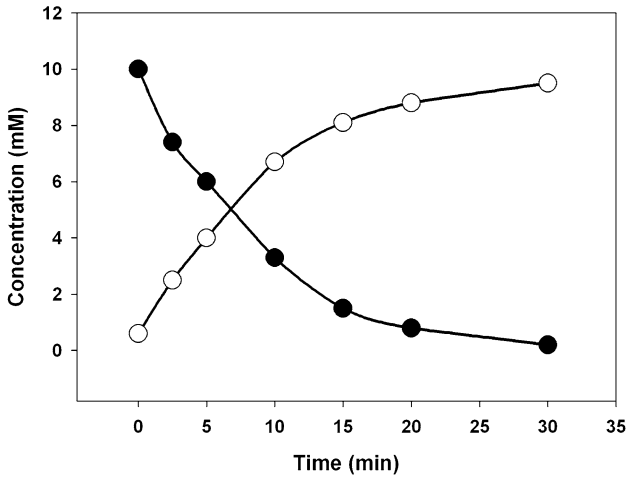

Fig. 5 Conversion of 2-hydroxy-3-butenenitrile by *E. coli* JM109(pIK9). Resting cells ($OD_{600\ nm}$ = 33) were incubated in K-phthalate buffer (50 mM, pH 6) at 6 °C with 10 mM 2-hydroxy-3-butenenitrile. The turn-over of 2-hydroxy-3-butenenitrile (●) to 2-hydroxy-3-butenoic acid (■) was quantified by HPLC

because of its symmetry also 2-butenediamide could be excluded as product).

The comparison of the observed chemical shifts with those previously determined for 3-cyanoacrylate and 3-cyanoacrylamide (Rey et al. 2004) demonstrated that the minor product (which showed more down-field shifted signals) was 3-cyanoacrylamide and that the major product was 3-cyanoacrylic acid (Fig. 7).

Influence of different mutations on the reaction specifity during the conversion of fumarodinitrile

It was previously shown for the conversion of mandelonitrile that variants of NitA could be obtained which converted the nitrile either to increased amounts of mandelic acid or mandeloamide (Kiziak and Stolz 2009; Sosedov and Stolz 2015). Therefore, it was tested if these variants also demonstrated different reaction specifities for the conversion of fumarodinitrile. Resting cells of *E. coli* JM109(pIK9/Ala165Phe) (forming increased amounts of mandelic acid from mandelonitrile; Kiziak and Stolz 2009) or *E. coli* JM109(pIK9/Trp188Lys) (forming increased amounts of mandeloamide from mandelonitrile; Sosedov and Stolz 2015) were incubated with fumarodinitrile and the reactions analyzed by HPLC.

Thus it was found that the variant NitA/Ala165Phe converted fumarodinitrile indeed to an increased proportion of

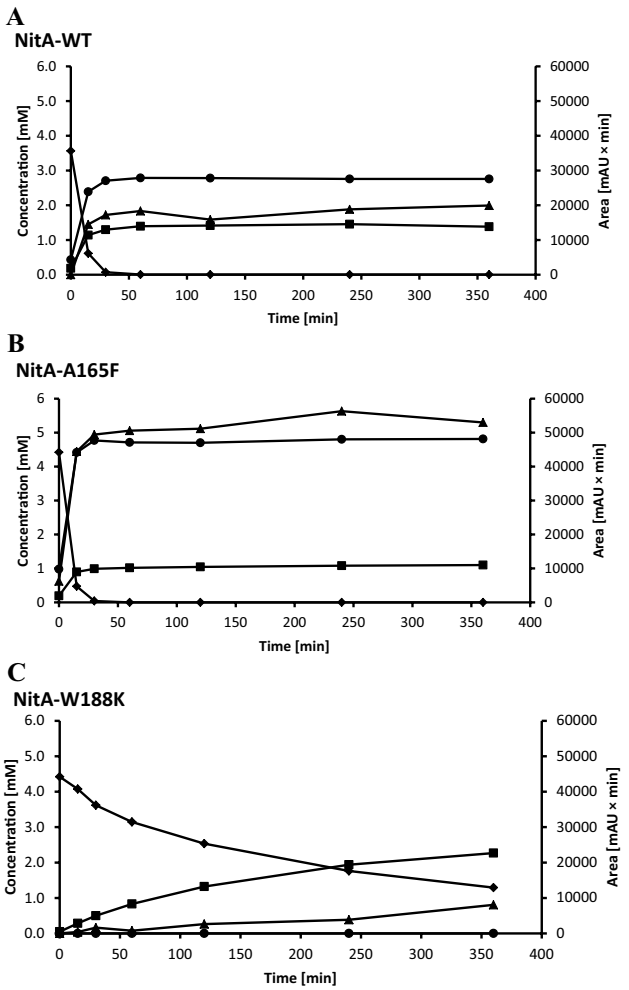

Fig. 6 Conversion of fumarodinitrile by NitA and the enzyme variants NitA(Ala165Phe) and NitA(Trp188Lys). The reaction mixtures contained in 0.81 ml 25 mM Tris/HCl (pH 7.5) and resting cells of *E. coli* JM109 synthesizing **A** NitA, **B** NitA(Ala165Phe) or **C** NitA(Trp188Lys) with optical densities ($OD_{600\ nm}$) of 30, 28, and 138, respectively. The reactions were started by the addition of fumarodinitrile (90 µl from a 50 mM stock solution in methanol). At the indicated time intervals, aliquots (90 µl each) were taken and the cells removed by centrifugation in an Eppendorf centrifuge (14,000 rpm, 2 min). The supernatants were analysed by HPLC and the concentrations of fumarodinitrile (◆) (R_t = 11.0 min) and the two metabolites 3-cyanoacrylamide (■) (R_t = 10.0 min) and 3-cyanoacrylic acid (●) (R_t = 13.0 min) quantified using a detector wavelength of 210 nm. The ammonia concentration (▲) was determined by using the assay described by Black et al. (2015). The concentrations of fumarodinitrile and ammonia are given in mM, those of the metabolites in relative units (due to the lack of commercially available standards)

3-cyanoacrylate (ratio 3-cyanoacrylate:3-cyanoacrylamide: about 80:20) (Fig. 6B).

The NitA/Trp188Lys variant converted fumarodinitrile only with low activities. This variant almost exclusively

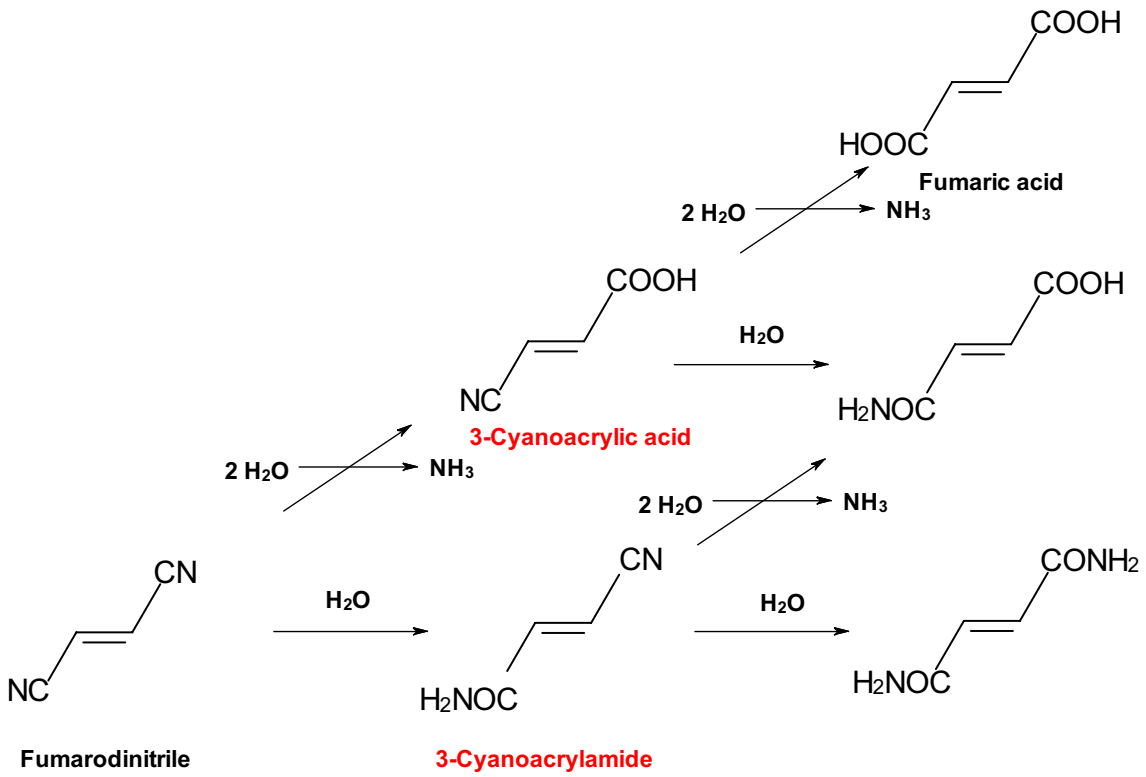

Fig. 7 Possible reaction products of fumarodinitrile

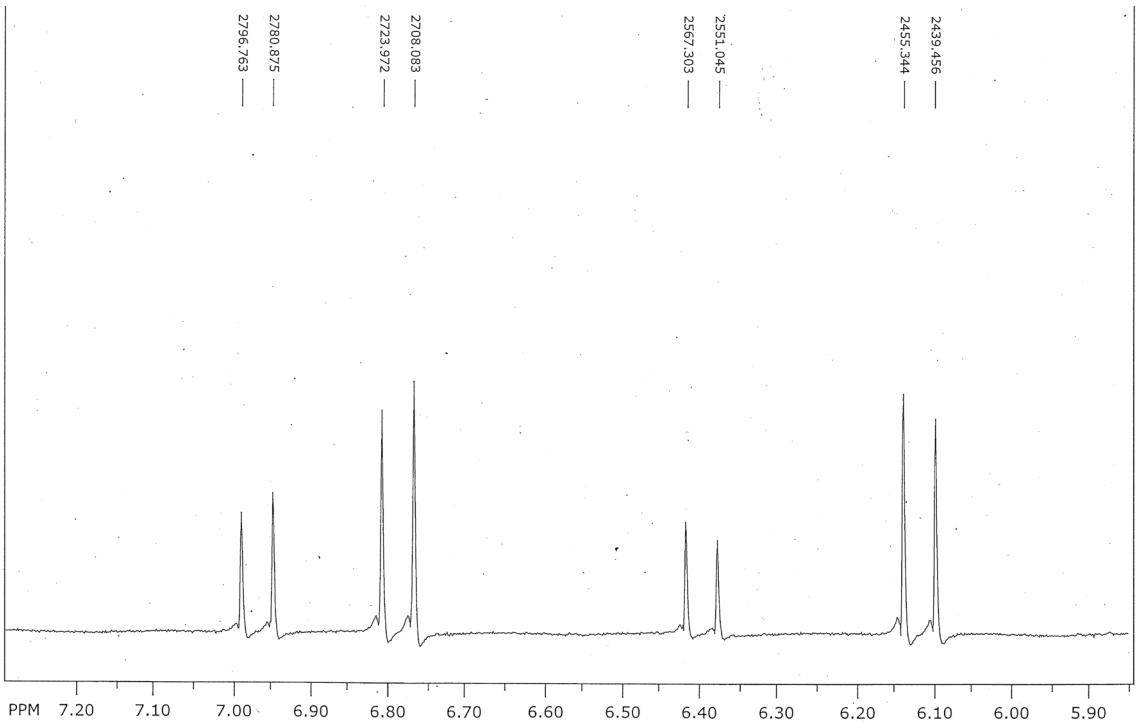

Fig. 8 Downfield region of 400 MHz ^{1}H nuclear magnetic resonance spectrum of the reaction mixture obtained after the complete conversion of fumarodinitrile by resting cells of *E. coli* (pIK9)

formed 3-cyanoacrylamide and no 3-cyanoacrylic acid could be detected (Fig. 6C).

Conversion of 3-hexenedinitrile and 2-methyleneglutarodinitrile

The initial screening experiments (Table 2) demonstrated that NitA converted in addition to fumarodinitrile also several C6-dinitriles with significant rates. Therefore, the turnover of 3-hexenedinitrile and 2-methyleneglutarodinitrile was further studied. The HPLC analysis demonstrated that 3-hexenedintrile ($R_t = 8.6$ min) was converted to only one detectable product ($R_t = 7.0$ min). In order to identify this product, a cell suspension of *E. coli* JM109(pIK9) ($OD_{600\ nm} = 10.8$) was incubated with an increased concentration of 3-hexenedinitrile (100 mM in 25 mM Na-phosphate, pH 7.5). After the complete conversion of the substrate (t = 100 min; detected by HPLC), the cells were removed by centrifugation, the supernatant spiked with D_2O (10% v/v) and directly analysed by ^{13}C NMR. Thus, it was found that the initially present 3 signals caused by (the symmetric) 3-hexenedinitrile [δ_c 19.50 ppm (NC–$\underline{C}H_2$–), 118.95 ppm (N$\underline{C}$–), 123.08 ppm (–CH_2–$\underline{C}H$=)] completely disappeared and were replaced by 6 new signals. This demonstrated that an unsymmetric product was formed, which could be identified (in accordance with the results obtained for the release of ammonia) as 5-cyano-3-pentenoic acid [δ_c 19.70 ppm (NC–$\underline{C}H_2$–), 40.86 ppm (HOOC–$\underline{C}H_2$–) 119.70 ppm (N$\underline{C}$–), 120.17 ppm (NC–CH_2–$\underline{C}H$=), 129.91 ppm (HOOC–CH_2–$\underline{C}H$=), 180.31 ppm (HOO$\underline{C}$–)].

2-Methyleneglutarodinitrile ($R_t = 8.9$ min) was converted to a major product ($R_t = 7.5$ min) and a minor product ($R_t = 6.6$ min) in a ratio (determined at $\lambda = 190$ nm) of about 10:1. The reaction was therefore also analysed by ^{13}C NMR (as described above for the conversion of 3-hexenedinitrile). The obtained spectrum confirmed the formation of two products and the turn-over of the signal caused by –$\underline{C}N$ (δ_c 134.89 ppm) to signals at δ_c 175.09 ppm and δ_c 180.70 ppm, probably caused by a –$\underline{C}OOH$– and a $\underline{C}ONH_2$-group. The formation of the organic products corresponded with the simultaneous release of almost stoichiometric amounts ammonia. These results indicated that 2-methyleneglutarodinitrile was hydrolysed mainly to the corresponding monocarboxylic, but that also some traces of the monoamide were formed.

Analysis of a specific amino acid residue that might be responsible for the ability of the nitrilase from *Pseudomonas fluorescens* EBC191 to convert aliphatic nitriles

It was previously described for the nitrilase from *Rhodococcus rhodochrous* ATCC 33278 that the exchange of Tyr142 against a non-aromatic amino acid residue resulted in an enzyme variant which still converted aromatic nitriles but was unable to convert aliphatic nitriles (Yeom et al. 2008). The importance of this residue for the substrate specificity of nitrilases occurred rather curious as multiple sequence alignments demonstrated that aromatic amino acid residues are highly conserved at this position among putative aromatic, aliphatic and arylacetonitrilases. Furthermore, a homology model of NitA suggested that the relevant tyrosine residue (Tyr141) is located rather far away from an enzyme bound aliphatic nitrile (Fig. 9A). Therefore, Tyr141 was modified in NitA by site-directed mutagenesis and the variants Tyr141Ala, Tyr141Phe, Tyr141Trp, and Tyr141His generated. Subsequently, these variants were analysed for their activities towards 2-phenylpropionitrile and valeronitrile. These experiments demonstrated that the Tyr141Ala variant was still active and converted the aliphatic and the aromatic substrate with similar relative activities as the wild-type enzyme (Table 3). Surprisingly, the most pronounced effect was found for the Tyr141Trp variant. This variant converted

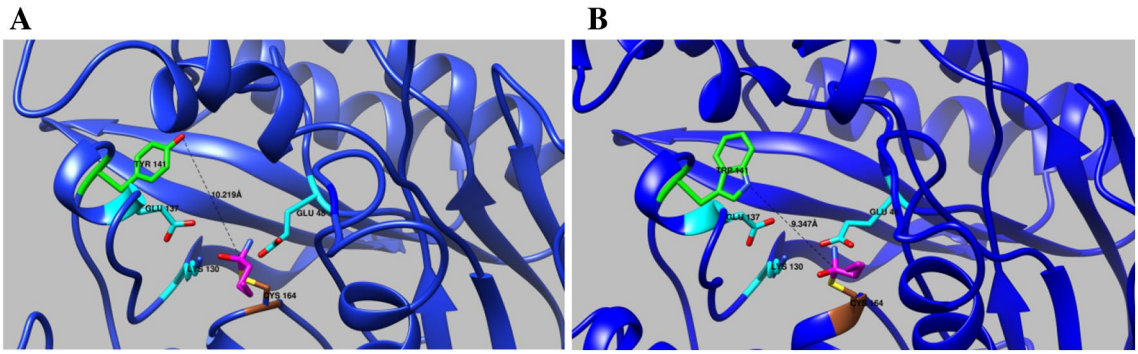

Fig. 9 Models of the catalytic center of the wild-type of NitA (**A**) and the variant NitA/Tyr194Trp (**B**) with a covalently bound tetrahedral intermediate of valeronitrile. The model was generated by homology modelling based on the structure of the nitrilase from *Synechocystis* sp. PCC6803 (PDB 3WUY) (Zhang et al. 2014)

Table 3 Specific activities of resting cells of *E. coli* JM109(pIK9) and derivatives carrying mutations in the nitrilase gene with 2-phenylpropionitrile and valeronitrile

Type of NitA	Substrate	Specific activity	Relative activity (Valeronitrile/2-PPN)	Relative activity for 2-PPN (in relation to the wild-type)
Wild-type	2-PPN	2.33 ± 0.02	0.29	1.0
	Valeronitrile	0.67 ± 0.02		
Tyr141Ala	2-PPN	1.34 ± 0.1	0.21	0.58
	Valeronitrile	0.29 ± < 0.01		
Tyr141Phe	2-PPN	1.45 ± 0.02	0.44	0.62
	Valeronitrile	0.64 ± < 0.01		
Tyr141Trp	2-PPN	0.02 ± < 0.01	10.5	0.01
	Valeronitrile	0.21 ± 0.01		
Tyr141His	2-PPN	2.60 ± 0.23	0.10	1.12
	Valeronitrile	0.27 ± 0.03		

E. coli JM109(pIK9) and its variants were grown and the nitrilase activitities induced as described in the materials and methods section. The cells were washed and suspended in Tris/HCl-buffer (50 mM, pH 7.5). The cell suspensions (10.8 ml) were transferred to 100 ml Erlenmeyer flasks and shaken at 30 °C with 100 rpm. The reactions were started by the addition of the nitriles (16.7 mM each) and samples taken at t = 0, 30, 60, 120, 180 and 300 min. Each reaction was performed twice

valeronitrile with a much higher relative activity than 2-phenylpropionitrile (Table 3).

Discussion

The first bacterial and fungal nitrilases which were discovered by Harper (1977a, b) preferentially hydrolysed benzonitrile and other aromatic nitriles, in which the nitrile group is directly attached to an aromatic nucleus. This class of nitrilases is in our days usually entitled as "aromatic" nitrilases. Later, a novel type of nitrilase was described from *Alcaligenes faecalis* JM3, which hydrolysed several arylacetonitriles with high specific activities (> 100 U/mg protein) but was unable to convert benzonitrile. For this type of nitrilase the term "arylacetonitrilase" has been coined (Nagasawa et al. 1990). In the same year, the conversion of aliphatic nitriles by a nitrilase from *Rhodococcus rhodochrous* K22 was described by Kobayashi et al. (1990a, b) and it was shown that this nitrilase converted certain aliphatic substrates, such as acrylonitrile, succinonitrile or glutaronitrile with higher relative activities than all tested aromatic nitriles (such as benzonitrile, several substituted benzonitriles or cyanopyridines). The concept of the existence of aliphatic nitrilases was subsequently taken over by other authors who isolated nitrilases with the ability to hydrolyse aliphatic nitriles or performed computer assisted sequence comparisons (Bayer et al. 2011; Cai et al. 2014; Gavagan et al. 1999; Heinemann et al. 2003a; Kim et al. 2009; Lévy-Schil et al. 1995; Sharma et al. 2018; Zhu et al. 2008).

The nitrilase from *P. fluorescens* EBC191 represents a typical arylacetonitrilase as it hydrolyses several arylacetonitriles with rather high specific activities and shows almost no activity with benzonitrile. Thus, for the purified enzyme specific activities with phenylacetonitrile, mandelonitrile, 2-phenylpropionitrile and benzonitrile of 68, 33, 4.1, and 0.1 U/mg of protein, respectively, have been determined (Kiziak et al. 2005). In the present study it was demonstrated that resting cells of *E. coli* JM109(pIK9) converted aliphatic nitriles such as 2-chloropropionitrile or valeronitrile with specific activities of 3.3 and 2.8 U/mg protein. The nitrilase constitutes in induced cells of *E. coli* JM109(pIK9) about 20% of the soluble proteins (Kiziak et al. 2005). Thus, it can be calculated that the pure nitrilase from *P. fluorescens* EBC191 shows with valeronitrile and 2-chloropropionitrile specific activities of > 10 U/mg of protein. A comparison of the specific activities found with NitA for the conversion of 2-chloropropionitrile and valeronitrile with those previously reported for the conversion of other aliphatic substrates by "aliphatic nitrilases" demonstrated that for these enzymes in most cases only rather low specific activities (< 5 U/mg) have been reported (Table 4). This comparison illustrates that almost all nitrilases which have been described as aliphatic nitrilases show only rather low specific activities with their substrates. Furthermore, in many of these studies only very few non-aliphatic substrates have been tested. Thus, if NitA would previously had only been tested with the substrates used in the present study, it would have been described as aliphatic nitrilase.

It was previously proposed (mainly by comparisons of fungal nitrilases) that aromatic, aliphatic and arylacetonitrilases could be distinguished by specific amino acid motives in close neighbourhood to the catalytical active cysteine residue. In these comparisons it was suggested that aromatic nitrilases and arylacetonitrilases show a specific histidine residue in a CWE**H** motif (the C represents the catalytical active cysteine residue). In contrast,

Table 4 Comparison of the activities of several "aliphatic nitrilases" with various substrates

Substrate	*Rhodococcus rhodochrous* K22[a]	*Comamonas testosteroni*[b]	*Acidovorax faecalis* 72W[c]	*Arabidopsis thaliana* Nit 1[d]	*Arthrobacter aurescens* CYC705[e]	*Pseudomonas fluorescens* Pf-5[f] +	*Bradyrhizobium japonicum* USDA110 blr3397[g] +	*Bradyrhizobium japonicum* USDA110 bll6402[g] +	Metagenomic[h]	*Synechocystis* spp. PCC6803[i]	*Pseudomonas fluorescens* EBC191[j]
Acetonitrile	28	–	5	–	–	–	–	–	5	–	–
Propionitrile	13	6	2	–	–	4	–	–	5	33	0
Butyronitrile	18	–	0	–	–	2	15	3	5	110	3
Isobutyronitrile	7	–	0	–	–	–	–	–	–	–	<5
Valeronitrile	41	–	0	–	5	–	57	6	–	222	32
2-Chloropropionitrile	7	–	–	–	–	–	–	–	–	270	69
Acrylonitrile	348	23	–	–	–	5	–	–	0	68	6
Crotononitrile	100	–	0	–	–	–	4	3	7	–	> 1
Malonodinitrile	45	–	14	3	76	–	–	–	0	–	–
Succinodinitrile	271	–	9	14	100	89	–	–	76	–	–
Glutarodinitrile	345	–	10	40	–	–	–	–	100	–	–
Adipodinitrile	110	100	1	120	27	–	–	–	91	530	–
Pimelonitrile (C7-Dinitrile)	27	–	–	270	–	–	–	–	–		–
Suberonitrile (C8-Dinitrile)	21	–	–	330	–	–	–	–	–	590	–
Sebaconitrile (C10-Dinitrile)	16	–	2	–	–	42	–	–	–	480	–
2-Methylglutaronitrile	–	–	4	–	–	–	–	–	77	394	–
Fumaronitrile	–	–	100	–	–	100	–	–	–	12,000	6
1,4-Dicyano-2-butene	43	–	–	–	–	–	–	–	–	14	–
Benzonitrile	27	4	5	–	0	4	1	1	0	100	0.25
Phenylacetonitrile	27	–	0	–	0	5	100	100	–	52	1650
2-Phenylpropionitrile(2PPN)	–	–	–	–	–	–	<1	10	0	–	100
Mandelonitrile	–	–	–	–	2	0	4	460	0	–	800
CWEX	H	H	H	N	N	N	N	A–H*	N	H	A–H*
Specific activity (=100%)	0.7 U/mg	29 U/mg	1 U/mg	–	9 U/mg	1.9 U/mg	2.3 U/mg	5.3 U/mg	13.7 U/mg	0. 33U/mg	4.1 U/mg

aliphatic nitrilases would show a CWEN motif. Furthermore, in the extension of this motif a threonine-residue (CWEHTQ**T**) would be specific for aromatic nitrilases and that the two other types of nitrilases would carry a proline residue in this position (Veselá et al. 2016). Unfortunately, this suggestion seems not generally to be valid (at least for bacterial nitrilases). Thus, the "archaetypical" aliphatic nitrilase from *Rhodococcus rhodochrous* K22, and the aliphatic nitrilases from *Comamonas testosteroni* sp., *Synechocystis* sp. PCC6803, and *Acidovorax facilis* 72W carry at the relevant position the motif CWEH (Kobayashi et al. 1992b; Lévy-Schil et al. 1995; Chauhan et al. 2003; NCBI BAA10717.1). In contrast, a CWEN motif is found in nitrilases from *Arthrobacter (Paenarthrobacter) aurescens* CYC705, a "metagenomic aliphatic nitrilase", blr3397 from *Bradyrhizobium japonicum* (*diazoefficiens*) USDA 110, *Pseudomonas fluorescens* Pf-5 and the typical plant nitrilases (Table 4) (Zhu et al. 2008; Bayer et al. 2011; Cai et al. 2014; Kim et al. 2009).

A different proposal for the differentiation of aliphatic nitrilases was suggested by Yeom et al. (2008). The authors identified from sequence comparisons of some bacterial and plant nitrilases a specific aromatic amino acid residue which according to the authors would allow to distinguish aromatic and aliphatic nitrilases. The authors modified this residue (Tyr142) in the nitrilase from *Rhodococcus rhodochrous* ATCC 33278, which was able to convert the aliphatic substrates glutarodinitrile and adipodinitrile with slightly higher activities than the aromatic substrates benzonitrile, *m*-tolunitrile, and 2-cyanopyridine. The importance of the investigated residue was shown for the variant Tyr142Ala which lost the ability to convert the aliphatic substrates, but was still able to hydrolyse the aromatic nitriles. The principle importance of the homologous residue for nitrilase activity was later also demonstrated for the nitrilase from *Synechocystis* spp. PCC6803, but for this nitrilase the mutation Trp146Ala resulted in a complete loss of activity towards aliphatic and aromatic substrates (Zhang et al. 2014). In the present study it was found for the nitrilase from *P. fluorescens* EBC191 that the analogous mutation Tyr141Ala resulted in a nitrilase variant that is still able to convert aromatic and aliphatic substrates. Therefore, it can be deduced that also the nature of this residue is not generally determinative for the substrate specifity of nitrilases.

In conclusion, it can be deduced that from the available sequence data it is not possible to define a distinct group of "aliphatic nitrilases". Furthermore, for the majority of "aliphatic nitrilases" which only show low specific activities with aliphatic substrates, it might be necessary to test a broad range of structural diverse nitriles before claiming the existence of an aliphatic nitrilase.

Table 4 (continued)

– Not determined

CWEX—amino acid sequence in direct neighborhood to the catalytical active cysteine residue (see text), * these nitrilases do not possess a "CWEX-motif", but a "CAEH-motif"

[a]Kobayashi et al. (1990a)

[b]Lévy-Schil et al. (1995)

[c]Gavagan et al. (1999)

[d]Effenberger and Oßwald (2001)

[e]Cai et al. (2014)

[f]Kim et al. (2009)

[g]Zhu et al. (2008)

[h]Bayer et al. (2011)

[i]Heinemann et al. (2003a)

[j]Kiziak et al. (2005)

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