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Conversion of phenylglycinonitrile by recombinant *Escherichia coli* cells synthesizing variants of the arylacetonitrilase from *Pseudomonas fluorescens* EBC191

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Abstract

The conversion of phenylglycinonitrile (2-aminophenylacetonitrile) by *Escherichia coli* strains was studied, which recombinantly expressed the arylacetonitrilase (NitA) from *Pseudomonas fluorescens* EBC191 and different nitrilase variants with altered reaction specificities. The whole-cell catalysts which formed the wild-type nitrilase converted (*R*,*S*)-phenylglycinonitrile preferentially to (*S*)-phenylglycine with a low degree of enantioselectivity. A recombinant strain which formed a variant of NitA produced mainly (*S*)-phenylglycine amide from (*R*,*S*)-phenylglycinonitrile and a second variant showed an almost complete enantioconversion and produced (*R*)-phenylglycine and left (*S*)-phenylglycinonitrile. The microbial-produced (*S*)-phenylglycinonitrile was used to study the chemical racemisation of (*S*)-phenylglycinonitrile at alkaline pH values in order to establish a dynamic kinetic resolution of the substrate. Subsequently, the conversion of (*R*,*S*)-phenylglycinonitrile by the whole-cell catalysts was studied at a pH of 10.8 which allowed a sufficient racemisation rate of phenylglycinonitrile. Surprisingly, under these conditions, strongly increased amounts of (*S*)-phenylglycine were formed by the recombinant *E. coli* cells expressing the amide-forming nitrilase variant. The aminopeptidase PepA from *E. coli* was identified by the construction of a deletion mutant and subsequent complementation as responsible amidase activity, which converted (*S*)-phenylglycine amide to (*S*)-phenylglycine.

Keywords Nitrilase · Nitrile hydratase · Amidase · Aminopeptidase

Introduction

Organic nitriles with the general formula R-CN can be enzymatically converted by nitrilases or nitrile hydratases. Nitrilases are hydrolytic enzymes found in many bacteria, fungi and plants which convert nitriles to the corresponding carboxylic acids and ammonia. Nitrile hydratases are almost exclusively found in bacteria and hydrate nitriles to amides. There is a considerable interest in chemistry in the utilisation of nitrilases and nitrile hydratases for chemo-, regio- or enantioselective biotransformation reactions (Bhalla et al. 2018; Gong et al. 2017; Martinková and Křen 2010; Prasad and Bhalla 2010; Singh et al. 2006).

A very interesting group of nitriles for biotransformation reactions are α-aminonitriles because they can be easily prepared by the Strecker reaction (or related syntheses) from aldehydes, ammonia and cyanide. The classical Strecker synthesis couples the formation of the aminonitriles in aqueous media with a subsequent acid hydrolysis of the aminonitriles to the corresponding acids. This is one of the most efficient and straightforward methods for the synthesis of various amino acids. The disadvantage of the Strecker reaction lies in the fact that the reaction results in the formation of racemic products. Therefore, significant interest exists in the development of asymmetric variations of the Strecker synthesis and organo- and metal catalysts have been described which induce enantioselectivity into the Strecker reaction (often using *N*-substituted aminonitriles as substrates) (Gröger 2003; Kouznetsov and Galvis 2018; Ooi et al. 2006; Surendra et al. 2006; Wang et al. 2011; Zuend et al. 2009).

There are some reports which describe the enantioselective conversion of racemic α-aminonitriles by nitrile hydratases

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and nitrilases (Wang and Lin 2001; Wegman et al. 2000, 2001; Hensel et al. 2002; Ewert et al. 2008). In the majority of these studies, the conversion of (substituted) phenylglycinonitrile(s) [2-aminophenylacetonitrile(s)] was studied as (substituted) (*R*)-phenylglycine(s) and (*R*)-phenylglycine amide(s) are used as building blocks in the synthesis of various β-lactam antibiotics and are therefore important industrial products (Bruggink 2001). Thus, different nitrile hydratases forming *Rhodococcus*, *Pantoea* and *Klebsiella* strains were found which convert racemic phenylglycinonitrile to (*S*)-phenylglycine with rather high enantioselectivities. The conversion of phenylglycinonitrile was caused in all analysed cases by the action of nitrile hydratases demonstrating only a low degree of enantioselectivity in combination with highly (*S*) specific amidases. Thus, these processes can lead to highly enantioenriched (*R*)-(D)-phenylglycine amide and (*S*)-(L)(+)-phenylglycine (Wang and Lin 2001; Wegman et al. 2000, 2001; Hensel et al. 2002; Ewert et al. 2008).

More recently, a coupling of a nitrile hydratase with two different amidases with opposite enantiopreferences together with an α-amino-ε-caprolactam racemase has been described which allowed the formation of almost enantiopure small aliphatic (*R*)- or (*S*)-amino acids via dynamic kinetic resolution processes (Yasukawa et al. 2011).

There are also some reports about the biotransformation of α-aminonitriles by nitrilases. Initially, it was found that nitrilases from different *Rhodococcus*, *Acinetobacter* and *Aspergillus* strains could convert aliphatic 2-aminonitriles and also phenylglycinonitrile with some enantioselectivity to the corresponding amino acids (Bhalla et al. 1992; Choi and Goo 1986; Macadam and Knowles 1985). Later, Chaplin et al. (2004) described the screening of a large set of recombinant nitrilases for the conversion of (*R*,*S*)-phenylglycinonitrile and identified one nitrilase which could be used for the synthesis of (*R*)-phenylglycine with a fairly good *ee*-value (91%). Recently, the application of this enzyme for the conversion of (*R*,*S*)-phenylglycinonitrile has been further optimised (Kawahara and Asano 2018). Furthermore, the application of a recombinant *E. coli* strain was reported, which expressed an enantioselective nitrilase from *Sphingomonas wittichii* and hydrolysed (*R*,*S*)-phenylglycinonitrile to (*R*)-phenylglycine with *ee*-values up to 97% (Qiu et al. 2014).

We have analysed throughout the last years a nitrilase from *Pseudomonas fluorescens* EBC191 (Baum et al. 2012; Brunner et al. 2018; Fernandes et al. 2006; Heinemann et al. 2003; Kiziak et al. 2005, 2007; Kiziak and Stolz 2009; Layh et al. 1992; Sosedov et al. 2010; Sosedov and Stolz 2014, 2015). This enzyme converts various phenylacetonitriles, such as 2-hydroxyphenylacetonitrile (mandelonitrile), 2-acetoxyphenylacetonitrile (O-acetylmandelonitrile), 2-methylphenylacetonitrile (2-phenylpropionitrile) and also α,α-disubstituted phenylacetonitriles with moderate enantioselectivities to the corresponding α-(or α,α-)(di)substituted carboxylic acids and varying amounts of the corresponding amides. Also several heterocyclic and aliphatic nitriles are converted by this enzyme (Baum et al. 2012; Brunner et al. 2018; Fernandes et al. 2006; Heinemann et al. 2003; Kiziak et al. 2005; Layh et al. 1992). In the course of the initial screening of the range of substrates which are converted by the nitrilase from *P. fluorescens* EBC191, it was found that the enzyme also converted phenylglycinonitrile (Kiziak et al. 2005). During the last years, several variants of this nitrilase have been generated which demonstrate significantly changed reaction- and enantiospecificities with mandelonitrile, 2-phenylpropionitrile and also fumarodinitrile (Brunner et al. 2018; Kiziak et al. 2007; Kiziak and Stolz 2009; Sosedov et al. 2010; Sosedov and Stolz 2014, 2015). There is still significant interest to find an efficient way to convert racemic phenylglycinonitriles produced by the Strecker synthesis to stoichiometric amounts of enantiopure phenylglycines or phenylglycine amides (this has been once claimed as one of the "holy grails of biocatalysis"; Sheldon and van Rantwijk 2004). Therefore, in the present study, different nitrilase variants were analysed for the conversion of (*R*,*S*)-phenylglycinonitrile.

Materials and methods

Bacterial strains, plasmids and culture conditions *Escherichia coli* JM109 was used as standard host strain. The construction of plasmid pIK9, which encodes for the wild-type nitrilase (NitA) from *Pseudomonas fluorescens* EBC191, has been described previously (Kiziak et al. 2005). Plasmids pIK9/Ala165Phe and pIK9/Trp188Lys were generated from plasmid pIK9 by site-directed mutagenesis (Kiziak and Stolz 2009; Sosedov and Stolz 2015). The nitrilase genes were expressed in these constructs under the control of a rhamnose-inducible promoter (Stumpp et al. 2000).

The cultivation of the recombinant strains and the conditions for the expression of NitA and its variants have been described before (Kiziak et al. 2005).

Biotransformations The conversion of (*R*,*S*)-phenylglycinonitrile by resting cells was routinely determined in reaction mixtures (1 ml each) containing 100 mM of the relevant buffers, 10 mM nitrile and an appropriate amount of cells (~optical density (OD_{600nm}) 0.5–5). The stock solutions of (*R*,*S*)-phenylglycinonitrile (100 mM) were prepared in methanol. The reaction mixtures were incubated in a thermomixer at 750 rpm at 23 °C. After different time intervals, samples (100 μl each) were taken and the reactions were stopped by the addition of 1 M HCl (10 μl). The samples were centrifuged at 15,000*g* for 2 min and the supernatants were analysed using high-pressure liquid chromatography (HPLC).

Preparation of (*S*)-phenylglycinonitrile Resting cells of *E. coli* JM109(pIK9/Ala165Phe) were suspended in 9 ml K-phosphate buffer (100 mM, pH 6.0) to an OD_{600nm} of about 5. The cell suspension was transferred to a 250-ml Erlenmeyer flask and the reaction started by adding 1 ml of 100 mM (*R*,*S*)-phenylglycinonitrile (in methanol). The reaction mixture was incubated at room temperature and stirred at 500 rpm. After 30 min, the reaction was stopped by acidification with 1 ml of 1 M HCl. The cells were removed by centrifugation (20,000*g*, 2 min) and the supernatant lyophilised overnight. The remaining powder was resuspended directly before the experiments in 1 ml of water to give a solution of (*S*)-phenylglycinonitrile.

Racemisation of (*S*)-phenylglycinonitrile under alkaline conditions A preparation of (*S*)-phenylglycinonitrile (prepared as described above) was mixed with 100 mM Na-carbonate buffer (pH 10.8), the pH adjusted with 1 M NaOH and the reaction mixtures (150 μl each) were placed in 300 μl HPLC vials. The vials were placed in a HPLC autosampler at 23 °C. Aliquots (5 μl each) were withdrawn every 30 min using the HPLC injector and analysed directly by chiral HPLC.

Analytical methods The synthesis and turn over of (*R*,*S*)-phenylglycinonitrile was routinely quantified by HPLC. Two different HPLC methods were applied.

For the achiral HPLC analysis, a solvent system consisting of 5% (*v*/*v*) methanol, 1 g/l hexane-1-sulfonic acid sodium salt and 0.1% (*v*/*v*) formic acid in H_2O was applied. A Pro C18 AQ column (Trentec Analysetechnik, Rutesheim, Germany) at a flow rate of 1 ml/min was used. The average retention times (R_t) under these conditions were as follows: benzaldehyde R_t = 11.5 min, mandelonitrile R_t = 8.8 min, phenylglycinonitrile R_t = 15.1 min, phenylglycine R_t = 3.0 min and phenylglycine amide R_t = 6.5 min.

The separation of the enantiomers of phenylglycinonitrile, phenylglycine amide and phenylglycine was achieved on a Crownpak CR(+) column (Daicel). The mobile phase (flow rate 1 ml/min) consisted of 16.3 g/l of 70% perchloric acid in water. The column was at 35 °C. For the enantiomers of phenylglycine amide and phenylglycine, stable purchasable reference compounds were available. In the described chromatographic system, the average retention times were for (*R*)- and (*S*)-phenylglycine amide R_t = 3.2 and 4.4 min, and for (*R*)- and (*S*)-phenylglycine R_t = 3.7 and 5.6 min, respectively.

The commercially available racemic (*R*,*S*)-phenylglycinonitrile showed under the same conditions two signals at R_t = 6.7 and 7.3 min. It was deduced that the signal with the shorter retention time was due to (*R*)-phenylglycinonitrile because in the cases of phenylglycine amide and phenylglycine the respective (*R*)-enantiomers, both showed the shorter retention times. This interpretation was confirmed during the biotransformation experiments as the preferred formation of the (*R*)- or (*S*)-enantiomers of phenylglycine amide and/or phenylglycine could always be correlated in an enantioconservative way with the preferred disappearance of the signal for the respective enantiomer of phenylglycinonitrile.

The separated compounds were detected at a detector wavelength of 210 nm.

Construction of a Δ*pepA* variant of *E. coli* JM109 The deletion of *pepA* was conducted by the method of Datsenko and Wanner (2000). The sequence coding for a kanamycin resistance and flanked by FRT sites was amplified from plasmid pC01kanFRTa using the primers pepA-DW-fw (5′CTATCTGTAGCCACCGCCGTTGTCTTTAAGATTCAGGAGCGTAGTGCT TGTGTAGGCTGGAGCTGCTTCG-3′) and pepA-DW-rev (5′-GATAAGGCGTTCACGCCGCATCCGGCAATAACAGCCTTGCCTGACGCAACATATGAATATCCTCCTTAGTTC-3′). These primers added about 50-bp regions lying up- and downstream of the *pepA* sequence (underlined above). The PCR product was digested by *Dpn*I and gel-purified. Then *E. coli* JM109(pKD46) was grown for 5 h at 30 °C in LB medium supplemented with ampicillin (100 μg/ml) and arabinose (0.2%). After 5 h, 5 ml of cells were harvested and washed twice with ice-cold glycerin (10% (*v*/*v*) and finally resuspended in ice-cold glycerin. Subsequently, in a final volume of 100 μl, 500 ng of the gel-purified PCR product was added and the cells transformed by electroporation. Next, 1 ml of LB medium was added to the cell suspension and the cells were incubated for 2 h at 37 °C. Finally, 250 μl of the cell suspension was plated on LB agar supplemented with kanamycin (50 μg/ml) and incubated overnight at 37 °C.

Colonies growing on LB-kanamycin agar were tested for the deletion of *pepA* by PCR using primers pepA-fw (5′-CACAGAAGGACGTGCATTAC-3′) and pepA-rev (5′-CATGATGGTTTGCTGGAAGG-3′) followed by digestion of the PCR product by *Eco*RV. Positive colonies (showing the correct *Eco*RV digestion pattern) were further tested for the ability to grow on LB-ampicillin agar plates. Positive *E. coli* clones, which were Δ*pepA* and grew on LB-kanamycin but not on LB-ampicillin (thus no longer bearing pKD46), were purified on LB-kanamycin agar plates. Single colonies of *E. coli* JM109 Δ*pepA*::*kan* were grown in liquid LB medium with kanamycin for 4 h at 37 °C and 180 rpm. The cells were harvested, washed twice with ice-cold glycerin and electroporated with pCP20 (Cherepanov and Wackernagel 1995), resuspended in LB and incubated at 30 °C for 2 h. Then the cells were plated onto LB agar plates with ampicillin and the plates incubated at 30 °C overnight. The colonies were streaked on LB agar and incubated at 42 °C overnight in order to cure pCP20. The resulting colonies were tested for growth on ampicillin and kanamycin (at 30 °C) and the mutation verified by PCR using primers pepA-fw and pepA-rev.

Cloning of the gene coding for aminopeptidase A The coding sequence of *pepA* was amplified from *E. coli* JM109 using primers pepA-NdeI-fw (AAAAACATATGGAGT TTAGTGTAAAAAG) and pepA-BamHI-rv (AAAAAGGATCCTTACTCTTCGCCGTTAAAC), which introduced *Nde*I and *Bam*HI restriction sites up- and downstream of the *pepA* sequence (restriction sites underlined). The PCR product and pJOE2775 (Stumpp et al. 2000) were cut with *Nde*I and *Bam*HI, the fragments gel-purified and finally ligated, yielding plasmid pEN15.

Chemicals (*R*, *S*)-phenylglycinonitrile, (*R*)- and (*S*)-phenylglycine amide and (*R*)- and (*S*)-phenylglycine were supplied by Sigma-Aldrich, Fluorochem and Fluka, respectively.

Results

Conversion of (*R,S*)-phenylglycinonitrile at pH 6 by variants of the nitrilase from *Pseudomonas fluorescens* EBC191

It was previously shown that resting cells of *E. coli* JM109(pIK9), which synthesize the wild-type nitrilase (NitA) from *P. fluorescens* EBC191, converted racemic phenylglycinonitrile to phenylglycine and phenylglycine amide and that preferentially the (*S*)-enantiomers of the products were formed (Kiziak et al. 2005). During the last years, several variants of NitA have been described, which convert mandelonitrile (2-hydroxyphenylacetonitrile) and 2-phenylpropionitrile (2-methylphenylacetonitrile) with significantly changed enantioselectivities and/or degrees of amide formation (Kiziak et al. 2007; Kiziak and Stolz 2009; Sosedov et al. 2010; Sosedov and Stolz 2014, 2015). Therefore, it was analysed if these variants also demonstrated changes in the reaction- and enantiospecificity during the conversion of phenylglycinonitrile. The enzyme variants NitA(Ala165Phe) and NitA(Trp188Lys) were tested which converted (*R*,*S*)-mandelonitrile either to increased amounts of (*R*)-mandelic acid or to increased amounts of (*S*)-mandeloamide, respectively (Kiziak and Stolz 2009; Sosedov and Stolz 2015).

Resting cells of *E. coli* JM109(pIK9), *E. coli* JM109(pIK9/Ala165Phe) and *E. coli* JM109(pIK9/Trp188Lys) were incubated in 100 mM K-phosphate buffer (pH 6.0) with (*R*,*S*)-phenylglycinonitrile (10 mM) and the reactions analysed by HPLC. Thus, it was found that the resting cells of *E. coli* JM109(pIK9), *E. coli* JM109(pIK9/Ala165Phe) and *E. coli* JM109(pIK9/Trp188Lys) converted (*R*,*S*)-phenylglycinonitrile with specific activities of 0.88, 0.34 and 0.32 U/mg of protein, respectively. The analyses of the reactions by chiral HPLC demonstrated that *E. coli* JM109(pIK9) and *E. coli* JM109(pIK9/Trp188Lys) preferentially converted the (*S*)-enantiomer of phenylglycinonitrile. In contrast, *E. coli* JM109(pIK9/Ala165Phe) preferred the (*R*)-enantiomer of this substrate.

In order to compare the reaction- and enantiospecificities of the three types of NitA, the reactions were each analysed after about 30% conversion of the racemic substrate. Thus, for the wild-type enzyme, the previous results were confirmed and it was found that phenylglycinonitrile was mainly converted to (*S*)-phenylglycine with a low degree of enantioselectivity (ee = 42%). In addition, about 8% of phenylglycine amide with a surplus of the (*S*)-enantiomer (ee = 75%) was formed (Table 1).

The variant *E. coli* JM109(pIK9/Trp188Lys) produced almost exclusively phenylglycine amide from (*R*,*S*)-phenylglycinonitrile. In comparison to the wild type, there was an increase in the *ee*-value of the formed (*S*)-phenylglycine amide observed (Table 1).

The variant *E. coli* JM109(pIK9/Ala165Phe) demonstrated a decreased degree of amide formation compared to the wild type and formed about 3% phenylglycine amide from (*R*,*S*)-phenylglycinonitrile. In contrast to the wild-type enzyme, the variant NitA(Ala165Phe) almost exclusively formed (*R*)-phenylglycine (Fig. 1). Thus, this point mutation resulted in an almost complete stereoinversion of the reaction (Table 1).

Generation and racemisation of (*S*)-phenylglycino-nitrile

It is well known that α-aminonitriles, such as phenylglycinonitrile, are in a chemical equilibrium with the corresponding aldehyde, cyanide and ammonia. This has several implications for the intended biotransformation. On the one hand, racemic phenylglycinonitrile (and other α-aminonitriles) can be easily prepared by the Strecker reaction from an aldehyde, cyanide and ammonia. On the other hand, α-aminonitriles are notoriously unstable in aqueous systems. This might be contraproductive as it results in the decomposition of the substrate (Fig. 2). In the case of chiral substrates, the reversibility of the reaction also offers the potential to racemise the aminonitriles. This could allow a dynamic kinetic resolution and thus a complete conversion of a racemic α-aminonitrile to a single enantiomer of a product.

The racemisation of (*R*,*S*)-phenylglycinonitrile in aqueous solutions has been studied in some detail by Chaplin et al. (2004). They demonstrated that phenylglycinonitrile racemises under alkaline conditions and that at a pH of 10.5, a solution of (*S*)-phenylglycinonitrile completely racemised within 60 min. In contrast, at pH 8 and pH 9.5, the respective values were 13 h and 4 h, respectively.

In order to define the pH value required for a combination of a chemical racemisation with an enzymatic reaction in

Table 1. Conversion of (*R*,*S*)-phenylglycinonitrile by recombinant *E. coli* strains synthesising different variants of the nitrilase NitA from *P. fluorescens* EBC191

Parameter	Value		
	Wild type	Trp188Lys	Ala165Phe
ee Phenylglycine	42	35	93
(preferentially formed enantiomer)	(*S*)	(*S*)	(*R*)
Phenylglycine amide [%] [PGAA]/([PGA] + [PGAA]) [%] [2]	8	> 95	3
ee Phenylglycine amide[2]	75	93	> 95%
(preferentially formed enantiomer)	(*S*)	(*S*)	(*R*)
ee Mandelic acid	31	66	79
(preferentially formed enantiomer)	(*R*)	(*R*)	(*R*)
Mandeloamide [%] [MAA]/([MA] + [MAA]) [%][2]	16	94	< 0.5
ee Mandeloamide[2]	84	57	n.d.
(preferentially formed enantiomer)	(*S*)	(*S*)	
ee 2-Phenylpropionic acid	65	39	91
(preferentially formed enantiomer)	(*S*)	(*S*)	(*R*)
2-Phenylpropionamide [%] [2-PPAA]/([2-PPA] + [2-PPAA]) [%][2]	0.2	60	0.1
ee 2-Phenylpropionamide	7	78	100
(preferentially formed enantiomer)	(*R*)	(*S*)	(*R*)

PGAA phenylglycine amide, *PGA* phenylglycine, *MAA* mandelic acid amide, *MA* mandelic acid, *2-PPAA* 2-phenylpropionic acid amide, *2-PPA* 2-phenylpropionic acid

Data for the conversion of mandelonitrile and 2-phenylpropionitrile were taken from Kiziak and Stolz (2009), Sosedov et al. (2010) and Sosedov and Stolz (2015)

some more detail, a sample of (*S*)-phenylglycinonitrile was obtained by conversion of (*R*,*S*)-phenylglycinonitrile with *E. coli* JM109(pIK9/Ala165Phe). The formation of highly enriched (*R*)-phenylglycine by this strain correlated with the intermediate accumulation of (*S*)-phenylglycinonitrile (Fig. 2) and this process could be used to obtain enantioenriched preparations of (*S*)-phenylglycinonitrile (see "Materials and methods"). The (*S*)-phenylglycinonitrile (1.4 mM) obtained was incubated at pH 9.5, 10.0, 10.5 pH 9.5, 10.0, and 10.5 in 100 mM Na_2CO_3 buffer and the racemisation of (*S*)-phenylglycinonitrile analysed by chiral HPLC. Thus, at pH 9.5, 10.0 and 10.5 for (*S*)-phenylglycinonitrile, half-lives were determined of approximately 360 min, 180 min and 130 min, respectively (Fig. 3). Furthermore, it became evident that in addition to the racemisation of (*S*)-phenylglycinonitrile to the (*R*)-enantiomer, also a decomposition of phenylglycinonitrile to benzaldehyde took place and that the increase in the pH value also resulted in an increased formation of benzaldehyde.

Conversion of (*R,S*)-phenylglycinonitrile under alkaline conditions by *E. coli* JM109(pIK9)

The experiments described above suggested that in order to achieve a dynamic kinetic resolution, it is necessary to perform the biotransformation of (*R*,*S*)-phenylglycinonitrile at pH values > 10. Therefore, resting cells of *E. coli* JM109(pIK9) were incubated with (*R*,*S*)-phenylglycinonitrile at pH 6, 9, 10 and 10.8 and the decrease in the substrate concentration analysed by HPLC. This demonstrated that the nitrilase activities in the cells were still active at pH values > 10, but that the activities significantly decreased at increasing pH values. Thus, at a pH of 10.8, about 30% of the activity at pH 6 were recovered. The two variants *E. coli* JM109(pIK9/Ala165Phe) and *E. coli* JM109(pIK9/Trp188Lys) showed similar pH-dependent decreases in the relative reaction rates (Fig. 4).

The incubation of the amide-forming strain *E. coli* JM109(pIK9/Trp188Lys) with (*R*,*S*)-phenylglycinonitrile at alkaline pH values showed a peculiar increase in the (*S*)-phenylglycine concentration at alkaline conditions. No (*S*)-phenylglycine could be detected under slightly acidic (pH 6.0) conditions. In contrast, at pH 10.8, significantly increased amounts of (*S*)-phenylglycine amide and (*S*)-phenylglycine were formed (Fig. 4).

Subsequently, the stability of the whole-cell catalysts in the relevant buffer systems was tested. Therefore, resting cells of *E. coli* JM109(pIK9) were incubated without substrate in the buffers (K-phosphate buffer pH 7, Na-carbonate buffer pH 10 and pH 10.8, all 100 mM) for 0, 30, 60 and 90 min before the substrate was added and the reaction rates were determined. This indicated that the nitrilase activity in the cells was at pH values between pH 7 and pH 10 stable for several hours, but showed at pH 10.8 only a half-life of approximately 2 h.

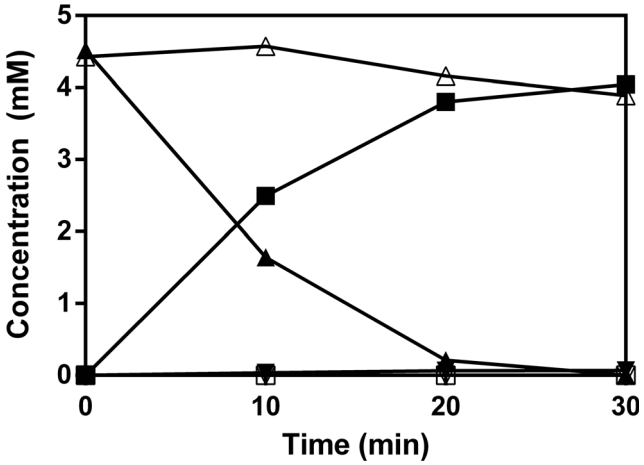

Fig. 1 Conversion of (*R*,*S*)-phenylglycinonitrile by resting cells of *E. coli* JM109(pIK9/Ala165Phe). The reaction mixture contained in 10 ml resting cells *E. coli* JM109(pIK9/Ala165Phe) (OD_{600nm} = 5) in 100 mM K-phosphate buffer (pH 6). The reaction was started by the addition of (*R*,*S*)-phenylglycinonitrile (10 mM). The concentrations of (*R*)-phenylglycinonitrile (▲), (*S*)-phenylglycinonitrile (△), (*R*)-phenylglycine amide (▼), (*S*)-phenylglycine amide (▽), (*R*)-phenylglycine (■) and (*S*)-phenylglycine (□) were monitored by chiral HPLC

Conversion of phenylglycine amide by *E. coli* JM109

The experiments with *E. coli* JM109(pIK9/Trp188Lys) demonstrated that at pH 6 from (*R*,*S*)-phenylglycinonitrile, almost stoichiometric amounts of phenylglycine amide were formed. In contrast, at alkaline pH values, significant amounts of phenylglycine were detected (Fig. 4). This could be due either to a chemical hydrolysis of phenylglycine amide under alkaline conditions or to a biological activity. The analysis of the biotransformation by chiral HPLC demonstrated that the phenylglycine which was formed under alkaline conditions was almost pure (*S*)-phenylglycine. Furthermore, it was found that only (*S*)-phenylglycine amide disappeared and the small amount of (*R*)-phenylglycine amide that was formed by the nitrilase variant was not converted. This indicated the presence of an enantioselective amidase activity in the whole-cell catalyst. Therefore, resting cells of *E. coli* JM109 and *E. coli* JM109(pJOE2775) ("vector control"; Stumpp et al. 2000) were incubated with (*R*,*S*)-phenylglycine amide in different buffer systems. These experiments demonstrated that the reference strains converted (*R*,*S*)-phenylglycine amide to (*S*)-phenylglycine and that the reaction rates increased with increasing pH values (Fig. 5).

Enantioselective hydrolysis of (*R*,*S*)-phenylglycine amide by the aminopeptidase PepA from *E. coli*

The hydrolysis of (*R*,*S*)-phenylglycine amide to (*S*)-phenylglycine by enantioselective amidases has been previously described for other bacteria, e.g. *Pseudomonas putida*, *Ochrobactrum anthropi* or *Mycobacterium neoaurum* (Hermes et al. 1993a, 1994; Sonke et al. 2005). In the case of *P. putida* ATCC 12633, this enzyme has been identified as an L-aminopeptidase which enantioselectively hydrolyses several dipeptides and L-amino acid amides (Hermes et al. 1993b). Three major aminopeptidases have been described for *E. coli*, peptidase A (PepA), peptidase B (PepB) and peptidase N (PepN) (Vogt 1970; Bhosale et al. 2010). Therefore, a BLAST search was performed with the sequence of the amidase/aminopeptidase from *P. putida* ATCC 12633 (CAA09054.1) against genomic sequences from *E. coli*. Thus, it was found that the highest scores were found with PepA (synonymously named leucyl aminopeptidase) from *E. coli* (53% sequence identity). Therefore, a Δ*pepA* variant of *E. coli* JM109 was generated by site-directed mutagenesis using the method described by Datsenko and Wanner (2000) as described in the "Materials and methods" section.

The variant *E. coli* JM109Δ*pepA* was transformed with plasmid pJOE2775 (="vector control") and plasmid pEN15 which carried the *pepA* gene cloned into pJOE2775. In the following, the turn over of (*R*,*S*)-phenylglycine amide by both strains was analysed. Thus, it was found that *E. coli* JM109Δ*pepA*(pJOE2775) showed only some residual activity for (*S*)-phenylglycine amide. In contrast, the complemented variant *E. coli* JM109Δ*pepA*(pEN15) converted (*S*)-phenylglycine amide with much higher reaction rates (Fig. 6). These experiments clearly demonstrated that PepA from *E. coli* is able to hydrolyze (*S*)-phenylglycine amide.

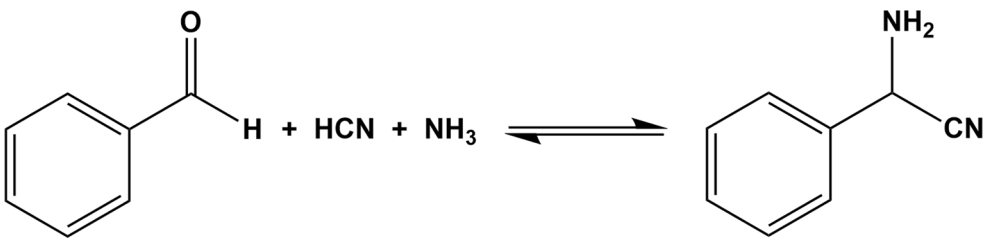

Fig. 2 Reversible formation of phenylglycinonitrile from benzaldehyde, cyanide and ammonia

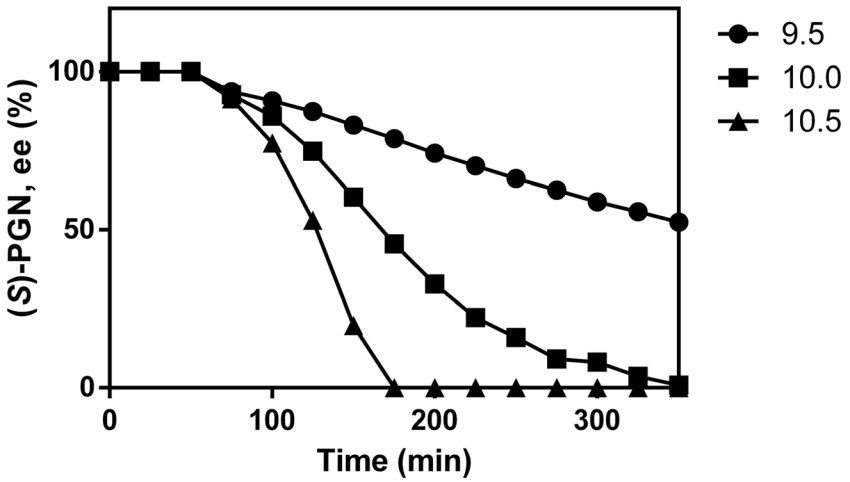

Fig. 3 Racemisation of (*S*)-phenylglycinonitrile at different pH values. The (*S*)-phenylglycinonitrile (PGN) was prepared with resting cells of *E. coli* JM109 (pIK9/Ala165Phe) as described in the "Materials and methods" section. This preparation was mixed (1:1, *v*:*v*) with 100 mM Na_2CO_3/$NaHCO_3$ buffer (pH 10.8) and brought to the indicated pH values by the addition of NaOH. The changes in the *ee*-values of (*S*)-phenylglycinonitrile were determined at pH, 9.5 (●), pH 10.0 (■) and pH 10.5 (▲) by HPLC

Discussion

The previous studies with the nitrilase from *P. fluorescens* EBC191 showed that by simple point mutations, variants could be generated which demonstrated with mandelonitrile and 2-phenylpropionitrile significantly changed reaction- and enantiospecificities (Kiziak et al. 2007; Kiziak and Stolz 2009; Sosedov et al. 2010; Sosedov and Stolz 2015). In the present study, it was shown that the observations previously made with nitriles which carry in the α-position a hydroxyl-

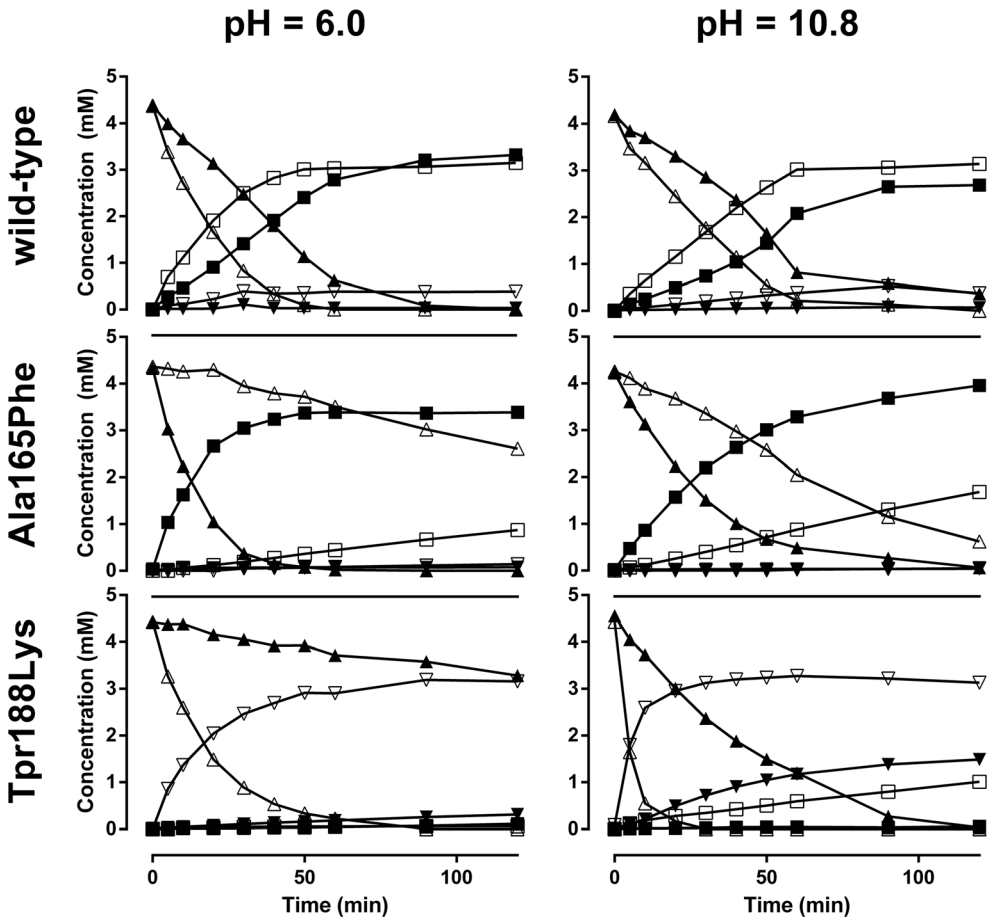

Fig. 4 Conversion of (*R*,*S*)-phenylglycinonitrile by resting cells of *E. coli* JM109(pIK9), *E. coli* JM109 (pIK9/Ala165Phe) and *E. coli* JM109 (pIK9/Trp188Lys) at different pH values. The reaction mixtures contained in a final volume of 1 ml resting cells of *E. coli* JM109 (pIK9) (OD_{600nm} = 0.1 for reactions at pH 6.0 and OD_{600nm} = 0.25 at pH 10.8), *E. coli* JM109(pIK9/Ala165Phe) or *E. coli* JM109(pIK9/Trp188Lys) (OD_{600nm} = 1.0 at pH = 6.0 and OD_{600nm} = 2.5 at pH 10.8) in 100 mM of K-phosphate- (pH 6.0) or 100 mM Na-carbonate buffer (pH 10.8). The reactions were started by the addition of 0.1 ml of 100 mM (*R*,*S*)-phenylglycinonitrile (in methanol). The concentrations of (*R*)-phenylglycinonitrile (▲), (*S*)-phenylglycinonitrile (△), (*R*)-phenylglycine (■), (*S*)-phenylglycine (□), (*R*)-phenylglycine amide (▼) and (*S*)-phenylglycine amide (▽) were determined by chiral HPLC

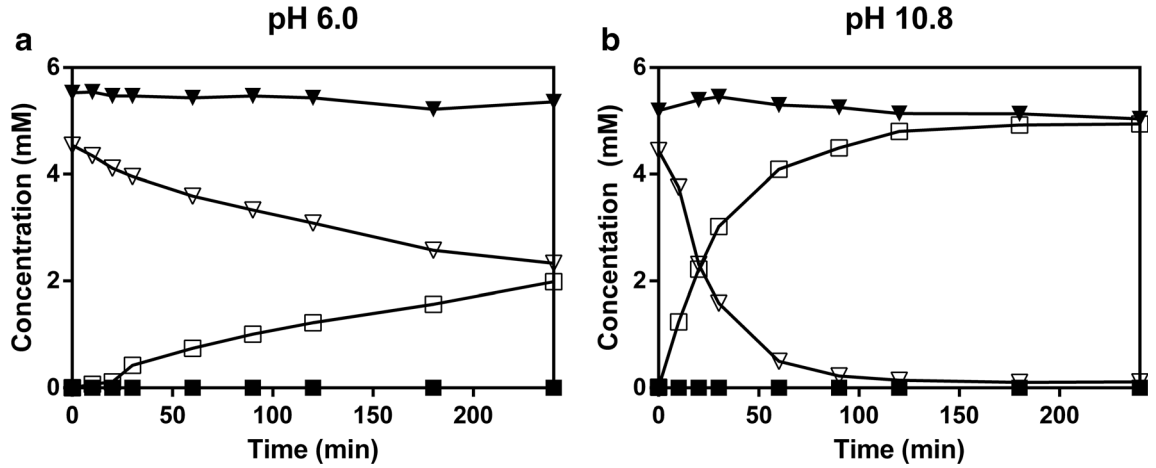

Fig. 5 Enantioselective hydrolysis of (*R*)- and (*S*)-phenylglycine amide by resting cells of *E. coli* JM109. The reaction mixtures contained in 10 ml resting cells of *E. coli* JM109 (OD_{600nm} = 10) in **a** 100 mM K-phosphate buffer (pH 6) or **b** 100 mM Na_2CO_3-buffer (pH 10.8). The reactions were started by the addition of (*R*)- and (*S*)-phenylglycine amide (5 mM each). The concentrations of (*R*)-phenylglycine amide (▼), (*S*)-phenylglycine amide (▽▽), (*R*)-phenylglycine (■) and (*S*)-phenylglycine (□) were measured by chiral HPLC

substituent (as in mandelonitrile) or a methyl-substituent (as in 2-phenylpropionitrile) can also be applied to substrates carrying an amino-function at the respective position. Thus, the nitrilase variant Ala165Phe (which carries in contrast to the wild-type enzyme a large substituent in direct neighbourhood to the catalytical active cysteine residue) demonstrated in comparison to the wild-type enzyme with mandelonitrile, a significantly enhanced (*R*)-selectivity and with 2-phenylpropionitrile and phenylglycinonitrile even an enantioconversion and formed instead of the (*S*)-enantiomers the respective (*R*)-enantiomers of the acids. Similarly, it was previously shown that the nitrilase variant Trp188Lys formed significantly increased amounts of amides from mandelonitrile and 2-phenylpropionitrile and in the present study it was established that this variant also forms a large surplus of phenylglycine amide from phenylglycinonitrile. The similar behaviour of the variants during the conversion of substrates carrying substituents with rather different polarity at the chiral centre suggested that the reaction- and enantiospecificity of these variants (and also of the nitrilase wild type) are largely governed by the size of the substituent.

The conversion of phenylglycine amide to (*S*)-phenylglycine has been found before with some Gram-positive and Gram-negative bacteria, such as *Mycobacterium neoaurum*, *Ochrobactrum anthropi* and *Pseudomonas putida* (Hermes et al. 1993a, 1993b, 1994; Sonke et al. 2005), but to the best of our knowledge, this reaction has not been previously described for *E. coli*. In the present study, it was clearly

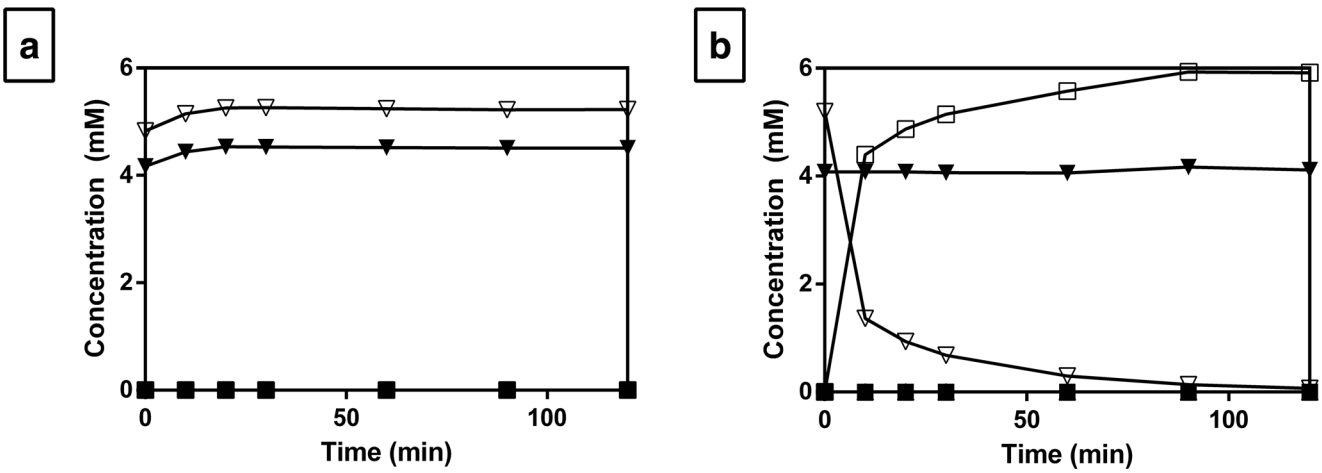

Fig. 6 Conversion of (*R*,*S*)-phenylglycine amide by resting cells of *E. coli* JM109 Δ*pepA*(pJOE2775) and *E. coli* JM109 Δ*pepA*(pEN15). The reaction mixtures contained in 1 ml resting cells of **a** *E. coli* JM109 Δ*pepA* (pJOE2775) (OD_{600nm} = 5.0) and **b** *E. coli* JM109 Δ*pepA*(pEN15) (OD_{600nm} = 1.0) in 100 mM Na-carbonate buffer (pH 10.8). The reactions were started by the addition of 5 mM (*R*)-phenylglycine amide plus 5 mM (*S*)-phenylglycine amide. The concentrations of (*R*)-phenylglycine (■), (*S*)-phenylglycine (□), (*R*)-phenylglycine amide (▼) and of (*S*)-phenylglycine amide (▽▽) were measured by chiral HPLC

shown by the construction of deletion mutants and the subsequent complementation of these mutants that the aminopeptidase PepA from *E. coli* is able to hydrolyse (*R*,*S*)-phenylglycine amide to (*S*)-phenylglycine with a high degree of enantioselectivity. This aminopeptidase has been previously purified and characterised by Vogt (1970). It was found that the enzyme hydrolysed several di- and tripeptides and that tripeptides such as Met-Leu-Gly or Met-Gln-Gly were the preferred substrates. Furthermore, the aminopeptidase was optimally active in the pH range from pH 9–11 and almost inactive at pH 7. The extraordinary pH optimum of the aminopeptidase PepA added an interesting new twist to the conversion of (*R*,*S*)-phenylglycine amide by genetically modified *E. coli* strains with the ability to convert (*R*,*S*)-phenylglycinonitrile to phenylglycine amide. Thus, it was possible to produce with *E. coli* JM109(pIK9/Trp188Lys) at pH 6 (*S*)-phenylglycine amide with ee-values > 90%, but the simple change in the pH to pH> 10 allows the synthesis of (*S*)-phenylglycine even with a higher enantiomeric excess as the aminopeptidase activity with its pronounced (*S*)-selectivity further "polishes" the enantiocomposition of the product.

Compliance with ethical standards

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

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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