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Biotransformation of benzonitrile herbicides via the nitrile hydratase–amidase pathway in rhodococci

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Abstract The aim of this work was to determine the ability of rhodococci to transform 3,5-dichloro-4-hydroxybenzonitrile (chloroxynil), 3,5-dibromo-4-hydroxybenzonitrile (bromoxynil), 3,5-diiodo-4-hydroxybenzonitrile (ioxynil) and 2,6-dichlorobenzonitrile (dichlobenil); to identify the products and determine their acute toxicities. *Rhodococcus erythropolis* A4 and *Rhodococcus rhodochrous* PA-34 converted benzonitrile herbicides into amides, but only the former strain was able to hydrolyze 2,6-dichlorobenzamide into 2,6-dichlorobenzoic acid, and produced also more of the carboxylic acids from the other herbicides compared to strain PA-34. Transformation of nitriles into amides decreased acute toxicities for chloroxynil and dichlobenil, but increased them for bromoxynil and ioxynil. The amides inhibited root growth in *Lactuca sativa* less than the nitriles but more than the acids. The conversion of the nitrile group may be the first step in the mineralization of benzonitrile herbicides but cannot be itself considered to be a detoxification.

Keywords Nitrile hydratase · Amidase · Benzonitrile herbicides · *Rhodococcus erythropolis* · *Rhodococcus rhodochrous*

Abbreviations

NHase	Nitrile hydratase
L-NHase	Low molecular weight NHase
H-NHase	High molecular weight NHase

Introduction

Benzonitrile herbicides have been known since the 1960s and their physico-chemical and biological properties (but mainly degradation pathways) were recently reviewed [10]. Bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) as a selective agent for the management of broad-leaved weeds in cereal crops is currently being applied to large areas of land. It will probably be used even more intensively in the future as a substitute for atrazine, which is being banned in an increasing number of countries. Ioxynil (3,5-diiodo-4-hydroxybenzonitrile) has a similar usage level to bromoxynil. On the other hand, dichlobenil (2,6-dichlorobenzonitrile) was used for the treatment of smaller areas such as private gardens, orchards, plant nurseries, or paths (*ibid.*). Its common metabolite 2,6-dichlorobenzamide is still contaminating groundwater even after the use of this herbicide was abolished in some countries [9]. The formation of this recalcitrant metabolite raised concerns as it

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is more mobile and hence more prone to spreading in the environment than the parent compound. Moreover, its biological effects have not yet been fully determined [9]. Though it has been shown that a slow mineralization of the benzonitrile herbicides occurs in soils [10], little is known about the organisms participating in this process.

Nitrilase-catalyzed hydrolysis of benzonitrile herbicides (except for dichlobenil) was found in a few microorganisms. A nitrilase that was highly specific for bromoxynil and ioxynil and their chlorinated analogue chloroxynil (3,5-dichloro-4-hydroxybenzonitrile) was described in *Klebsiella pneumoniae* ssp. *ozaenae* [15, 29]. The corresponding gene was used to construct resistant plants [7]. Rhodococci exhibited much lower nitrilase activities for benzonitrile herbicides [8, 32]. Nevertheless, these activities were sufficient to eliminate 0.5 mM chloroxynil, bromoxynil, or ioxynil from the culture medium [32].

The transformation of benzonitrile herbicides by nitrile hydratase (NHase) was reported in *Rhizobium radiobacter* (formerly *Agrobacterium radiobacter*; [9, 35]) and *Variovorax* sp. [17]. In contrast to the nitrilase producers, these organisms not only transformed 4-hydroxybenzonitrile analogues but also dichlobenil. The degradation of bromoxynil into the corresponding amide and acid in a flexibacterium [26] and a strain of *Pseudomonas putida* [34] indicated the participation of the same enzyme, followed by an amidase. The hydration of dichlobenil was also reported in *Arthrobacter*, *Rhodococcus*, and *Pseudomonas* genera (for a review see [10]). In rhodococci, the dichlobenil degraders were *Rhodococcus erythropolis* strains DSM 9675, DSM 9685 [9] and AJ270 [27] (previously designated *Rhodococcus rhodochrous* AJ270 [16]).

In rhodococci, a transformation of 4-hydroxybenzonitrile analogues via the NHase-amidase pathway has not yet been described to our knowledge. In this work, we report that rhodococci with NHase activities are not only able to transform dichlobenil but also chloroxynil, bromoxynil, and ioxynil. Rhodococci form two variants of NHases differing in their cofactor (Fe^{3+} or Co^{3+}). The NHase in *R. erythropolis* AJ270 used for dichlobenil biotransformation (see above) was Fe-type [27], which is typical for this species [4] (for a review see [14]). In this study, two strains were used, that is *R. erythropolis* A4 [13] and *R. rhodochrous* PA-34 [3]. While the NHase in *R. erythropolis* A4 is of the Fe-type, as confirmed by gene sequencing [13], the NHase in *R. rhodochrous* was postulated to be of the Co-type, as indicated by the effect of cobalt ions on its activity [20] and by the size of its subunits [19]. In this work, we confirmed this hypothesis by partial sequencing of the corresponding genes. The strains were compared in terms of the biodegradation rates and the type of the reaction products some of which were also examined for their acute toxicities towards *Vibrio fischeri* and for their inhibition of root growth in *Lactuca sativa*.

Materials and methods

Chemicals

The substrates—benzonitrile, 3,5-dichloro-4-hydroxybenzonitrile (chloroxynil), 3,5-dibromo-4-hydroxybenzonitrile (bromoxynil), 3,5-diiodo-4-hydroxybenzonitrile (ioxynil) and 2,6-dichlorobenzonitrile (dichlobenil)—and the authentic standards of their biotransformations—benzamide, benzoic acid, 3,5-dichloro-4-hydroxybenzoic acid, 3,5-dibromo-4-hydroxybenzoic acid, 3,5-diiodo-4-hydroxybenzoic acid, 2,6-dichlorobenzamide, 2,6-dichlorobenzoic acid—were purchased from standard commercial sources (Sigma-Aldrich, Alfa Aesar) and were of analytical-grade purity. Authentic standards of 3,5-dichloro-4-hydroxybenzamide, 3,5-dibromo-4-hydroxybenzamide, 3,5-diiodo-4-hydroxybenzamide were purchased from Shanghai Fangkai Chemical (China).

Microorganisms

Rhodococcus erythropolis A4 [13] and *R. rhodochrous* PA-34 [3] are the soil isolates characterized previously. Our BLAST analysis of a part of the *R. rhodochrous* PA-34 16S rDNA (468 bp) showed that the strain PA-34 is more related to *R. ruber* (strain KNUC9051, Acc. No. JF505985, 2 mismatches, 99.57 % identity) than to *R. rhodochrous* (isolate B31, Acc. No. AJ565926, 14 mismatches, 97.01 % identity). The strains were maintained on meat peptone agar (in g/l Bacto beef extract 3, peptone 10, NaCl 5, agar 15) and periodically transferred to fresh media. *R. erythropolis* A4 was grown in 500-ml Erlenmeyer flasks with 100 ml of a mineral medium according to [5] with 10 g l^{-1} glycerol and 3 g l^{-1} yeast extract as C and N source, respectively. This medium was inoculated with a suspension of cells grown on meat peptone agar and cultivated at 30 °C for 48 h. *R. rhodochrous* PA-34 was grown in 500-ml Erlenmeyer flasks with 100 ml of M4 medium with 0.02 g l^{-1} $CoCl_2$ and 2 g l^{-1} propionitrile [20] at 30 °C for 36 h. Each flask was inoculated with 4 ml of a preculture prepared as described previously [32].

DNA manipulation and sequencing

Part of the *nhlB-nhlA* gene cluster (1,199 bp) was amplified by PCR using Pfx polymerase (Carlsbad, CA), the NHCoBETAF (CCGATGAACCTGTTTTCCAT) and NHCo ALPHAR (AACTTCGCTACTGGGTCCTG) primers and *R. rhodochrous* PA-34 genome DNA as a template. The fragment of 16S rDNA from PA-34 was amplified with 8F (AGAGTTTGATYMTGGCTCAG) and 1492R (TACGG YTACCTTGTTACGACTT) universal primers [30]. The DNA fragments were sequenced with an ABI PRISM 3100

automatic sequencer (Applied Biosystems). The common 518R primer (CGTATTACCGCGGCTGCTGG) [1] was used for sequencing of 16S rDNA. The same primers as those used for PCR amplification of the *nhlB-nhlA* fragment were applied for its sequencing.

Enzyme assays

When determining NHase activity, appropriately diluted cell suspensions (475 µl) were preincubated at 28 °C for 5 min and benzonitrile (25 µl of 500 mM stock solution in methanol) was added to a final concentration of 25 mM. After shaking at 850 rpm and 28 °C for 3 min, the reactions were stopped by adding 100 µl of 1 M HCl. The reaction mixtures were centrifuged (14,000 rpm, 5 min) and analyzed for their reaction products (benzamide and benzoic acid) by HPLC (see below). One unit of NHase activity was defined as the amount of enzyme that formed 1 µmol of total product (sum of benzamide and benzoic acid) from benzonitrile per min under the above conditions. Amidase activity was determined as described previously [32].

Biotransformations

At analytical scale, the biotransformations were carried out at 30 °C and with shaking (850 rpm) in 1.5-ml Eppendorf tubes with 600 µl of the reaction mixtures consisting of appropriately diluted whole-cell suspensions (570 µl) and the substrates (chloroxynil, bromoxynil, ioxynil, or dichlobenil; 30 µl from 10 mM stock solutions in methanol; final concentration 0.5 mM). A total of 200 µl of methanol was added to aliquots (100 µl) of the reaction mixtures to stop the reaction. After centrifugation, the concentrations of nitriles, amides, and carboxylic acids in the supernatants were determined by HPLC as described previously [32].

For preparative purposes, chloroxynil, bromoxynil, ioxynil, and dichlobenil (50 mg each) were dissolved in 2.5 ml of methanol and 97.5 ml of the cell suspension was added. The reactions proceeded in shaken 250-ml Erlenmeyer flasks at 200 rpm and 28 °C for 1 day (biotransformations of chloroxynil, bromoxynil, or ioxynil by strain A4) or 3 days (others). Cells were removed by centrifugation (6,000 rpm, 30 min, 4 °C). Reaction products were extracted from the supernatants with ethyl acetate at pH 2–2.5 (2 M HCl). Extracts were dried with Na_2SO_4, filtrated, and the solvent evaporated under reduced pressure. NMR and MS/MS spectra were recorded and analyzed as described previously [32]. Negative NALDI-TOF (nano-assisted laser desorption ionization-time of flight) mass spectra were measured in an ultraFLEX III mass spectrometer (Bruker-Daltonics, Bremen, Germany) with external calibration using the monoisotopic $[M–H]^-$ ions of the PepMixII calibrant (Bruker-Daltonics, Bremen, Germany).

NMR spectra of 3,5-dichloro-4-hydroxybenzoic acid, 3,5-dibromo-4-hydroxybenzoic and 3,5-diiodo-4-hydroxybenzoic acid (not shown) were in excellent agreement with the previous data [32]. The negative NALDI-TOF MS revealed *m/z* 204.58, 206.59 for 3,5-dichloro-4-hydroxybenzoic acid, *m/z* 292.58, 294.59, 296.59 for 3,5-dibromo-4-hydroxybenzoic acid, *m/z* 388.59 for 3,5-diiodo-4-hydroxybenzoic acid and *m/z* 188.63, 190.63 for 2,6-dichlorobenzoic acid.

Spectral data of amides are only displayed for products prepared using the *R. rhodochrous* PA-34 strain. The data obtained for products of the *R. erythropolis* A4 strain indicated the same structures.

3,5-Dichloro-4-hydroxybenzamide: ^{1}H NMR: 7.394 (1H, br s, NH_2), 7.881 (2H, s, H-2, H-6), 7.976 (1H, br s, NH_2); ^{13}C NMR: 121.98 (C-3, C-5), 126.81 (C-1), 128.18 (C-2, C-6), 151.77 (C-4), 165.45 (CO). Positive MS *m/z* 206.04, 208.07 $[M + H]^+$.

3,5-Dibromo-4-hydroxybenzamide: ^{1}H NMR: 7.376 (1H, br s, NH_2), 7.790 (1H, br s, NH_2), 8.055 (2H, s, H-2, H-6); ^{13}C NMR: 111.37 (C-3, C-5), 128.09 (C-1), 131.86 (C-2, C-6), 153.40 (C-4), 165.11 (CO). Positive MS *m/z* 294.19, 296.14, 298.10 $[M + H]^+$.

3,5-Diiodo-4-hydroxybenzamide: ^{1}H NMR: 7.296 (1H, br s, NH_2), 7.929 (1H, br s, NH_2), 8.240 (2H, s, H-2, H-6); ^{13}C NMR: 86.00 (C-3, C-5), 129.63 (C-1), 138.79 (C-2, C-6), 158.03 (C-4), 164.94 (CO). Positive MS *m/z* 390.12 $[M + H]^+$.

2,6-Dichlorobenzamide: ^{1}H NMR: 7.396 (1H, dd, $J = 8.8$, 7.4 Hz, H-4), 7.473 (1H, d, $J = 7.4$ Hz, H-3), 7.474 (1H, d, $J = 8.8$ Hz, H-5), 7.782 (1H, br s, NH_2), 8.064 (1H, br s, NH_2). The peaks of H-3 and H-5 might be interchanged. ^{13}C NMR: 128.07 (C-3, C-5), 130.61 (C-4), 130.74 (C-2, C-6), 137.10 (C-1), 165.32 (CO). Positive MS *m/z* 190.1, 192.1 $[M + H]^+$.

Toxicity test using *V. fischeri*

The toxicity tests were performed and EC_{50} values calculated as described previously [32, 33].

Toxicity test using *Lactuca sativa*

The effect of the tested compounds on the average root length of germinating seeds was determined as described previously [32].

Results

Identification of the genes encoding subunits of Co-type nitrile hydratase in *R. rhodochrous* PA-34

Using the sequences of the *nhlA* and *nhlB* genes encoding α and β subunits of Co-type NHase from *R. rhodochrous* J1

[11], the oligonucleotide primers NHCoBETAF and NHCoALPHAR complementary to internal sequences of the *nhlB-nhlA* cluster were designed and used for amplification of 1,199-bp fragment. The sequence of this fragment showed 99.7 % identity with the *nhlB-nhlA* DNA sequence from *R. rhodochrous* J1. The proteins coded by *nhlB* and *nhlA* from the J1 and PA-34 strains shared more than 99 % identity (data not shown). Attempts to amplify by PCR fragments of the *nha1* and *nha2* genes encoding Fe-type NHase using *R. rhodochrous* PA-34 genome DNA as a template and various primers based on the *nha1* and *nha2* genes from various strains of *R. erythropolis* failed (data not shown). We concluded, therefore, that *R. rhodochrous* PA-34 contains a low molecular weight NHase (L-NHase).

Transformation of dichlobenil in *R. rhodochrous* PA-34

Rhodococcus rhodochrous PA-34 was cultivated under conditions optimized for NHase production (induction with propionitrile [20]) and yielded approximately 3.8 U_{NHase} mg^{-1} dry cell weight for benzonitrile. The absence of benzoic acid in the reaction product indicated that the strain produced no significant nitrilase activity under these conditions in contrast to the previous study, in which it was grown with isobutyronitrile [32]. The resting cells (43 U_{NHase} ml^{-1}) slowly decreased the dichlobenil concentration in the reaction mixture, while the formation of a more polar product was detected by HPLC. This compound exhibited the same retention time and UV spectrum as an authentic standard of 2,6-dichlorobenzamide. The identity of the product was confirmed by NMR and MS experiments. After calibration with the authentic standard, the time course of the amide formation was monitored (Fig. 1a). After 5 h, 28 % of the nitrile was consumed and approximately half of this was converted into 2,6-dichlorobenzamide. After 1 and 3 days, the nitrile conversion increased to 60 and 89 %, respectively, while the amide was only formed with yields of 27 and 48 %, respectively (Table 1). The lower yield of amide compared to nitrile consumption may be caused by a further reaction of 2,6-dichlorobenzamide or a conversion of dichlobenil by other pathways but also by a precipitation of the poorly water-soluble substrate, as no other products were found by HPLC. In agreement with our previous findings [32], the amidase activity of this strain was very low. Irrespective of the cultivation procedure (induction with isobutyronitrile [32] or propionitrile) the activity of this enzyme for benzamide was only 0.013–0.014 U mg^{-1} dry cell weight at 45 °C. This is consistent with the observation that no conversion of 2,6-dichlorobenzamide into 2,6-dichlorobenzoic acid occurred.

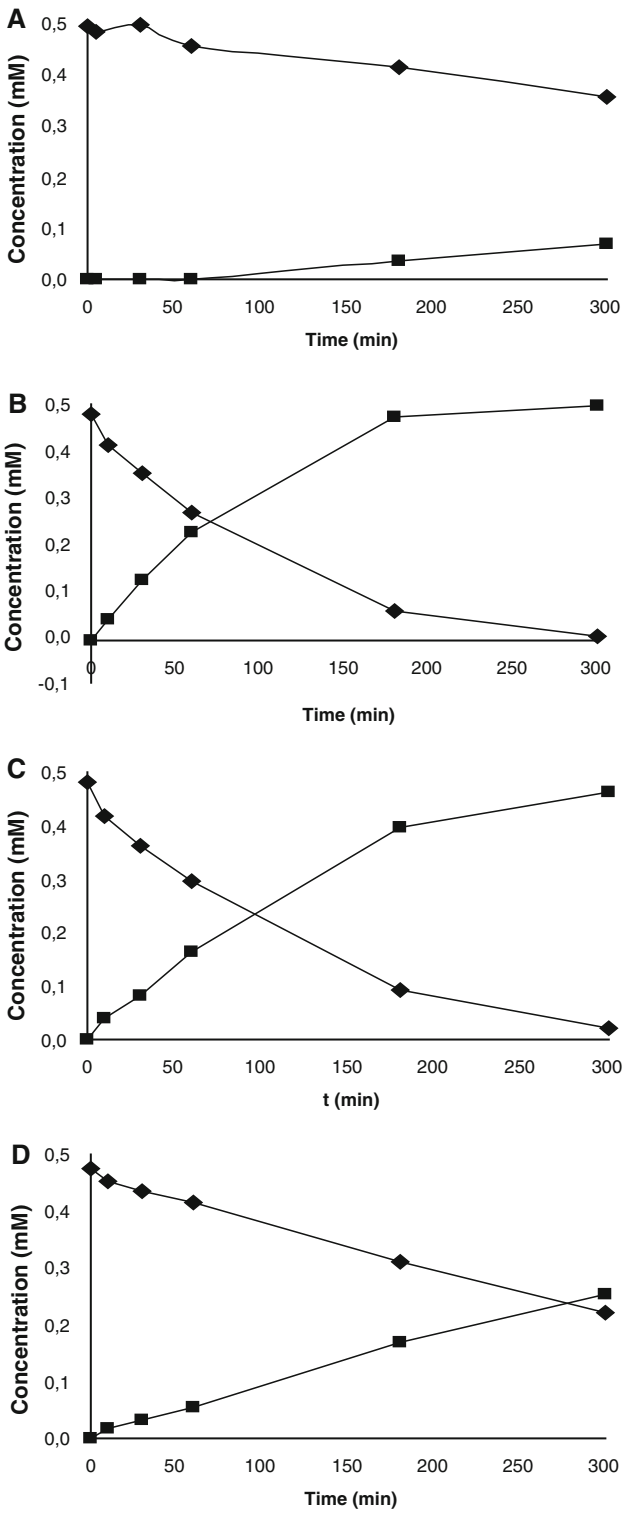

Fig. 1 Biotransformations of dichlobenil (**a**), chloroxynil (**b**), bromoxynil (**c**) and ioxynil (**d**) (0.5 mM each) by *R. rhodochrous* PA-34. Nitriles (*diamonds*), amides (*squares*). The reaction mixture ($OD_{610} = 40$) contained 43 U per ml of nitrile hydratase activity and 0.15 U per ml of amidase activity (determined for benzonitrile and benzamide, respectively)

Table 1 Products of benzonitrile analogue (0.5 mM) biotransformation

	Time (days)	Conversion (%)							
		Dichlobenil		Chloroxynil		Bromoxynil		Ioxynil	
		Amide	Acid	Amide	Acid	Amide	Acid	Amide	Acid
R. erythropolis A4	1	65	26	–	88	33	67	47	3
	3	40	41	–	80	27	61	42	3
R. rhodochrous PA-34	1	27	–	96	4	95	1	92	2
	3	48	–	86	14	98	2	90	10

The nitrile conversion was 100 % except for dichlobenil in the *R. rhodochrous* PA-34 strain (60 and 89 % after 1 and 3 days, respectively) and ioxynil in the *R. erythropolis* A4 strain (66 and 67 % after 1 and 3 days, respectively)

Transformation of dichlobenil in *R. erythropolis* A4

Rhodococcus erythropolis A4 cells exhibited a lower specific NHase activity for benzonitrile (1.0 U_{NHase} mg^{-1} dry cell weight) than the aforementioned strain. Therefore, the cell density was increased fourfold in strain A4, in order to achieve comparable enzyme loads (37 U_{NHase} ml^{-1}). *R. erythropolis* A4 transformed 0.5 mM dichlobenil faster than *R. rhodochrous* PA-34 (Figs. 2a and 1a, respectively), thus suggesting different substrate specificities of the NHases in these two strains. The same product was formed, 2,6-dichlorobenzamide, as indicated by the retention time and UV, NMR and MS spectra. The substrate was fully converted into this product, which remained unreacted in the reaction mixture after 5 h. However, after prolonged reaction times (1–3 days), the amide was partially converted into another product, which was identified by NALDI-TOF as 2,6-dichlorobenzoic acid and accounted for 50 % of the final total product (sum of amide and carboxylic acid; Table 1). As in the strain PA-34, the yield of the total product was lower than the theoretical yield (by 9 and 19 % after 1 and 3 days, respectively). The difference in the dichlobenil transformation patterns of the PA-34 and A4 strains coincided with the difference in their amidase activity, which was two orders of magnitude higher in the latter strain (4.2 U mg^{-1} dry cell weight for benzamide at 45 °C).

Transformation of 4-hydroxybenzonitrile analogues in *R. rhodochrous* PA-34

The biotransformations of all 4-hydroxybenzonitrile analogues were faster than those of dichlobenil in this strain (Fig. 1b–d). The reaction rates were slightly lower with bromoxynil than chloroxynil, both compounds being almost fully transformed within 5 h, and the lowest with ioxynil, which was only approximately 55 % converted within the same period. A single product was obtained from each biotransformation, isolated and identified as the corresponding amide. The yields of 4-hydroxybenzamides were close to or equal to theoretical yields, suggesting that any side reactions of the substrate or product were not significant. Prolonged incubation (1–3 days) resulted in only a slight decrease in the concentrations of the amides, which were partially hydrolyzed into carboxylic acids (Table 1).

Transformation of 4-hydroxybenzonitrile analogues in *R. erythropolis* A4

In the *R. erythropolis* A4 strain, the reaction rate of chloroxynil (Fig. 2b) was significantly higher than in *R. rhodochrous* PA-34 (Fig. 1b). The reaction rates of the other two compounds (Fig. 2c, d) were similar to those in the PA-34 strain. Almost all chloroxynil and bromoxynil was transformed within 1 and 3 h, respectively, but ioxynil was approximately only half-transformed within that time. The primary products of the biotransformations were the same for both strains, but, in *R. erythropolis* A4, the amides obtained from chloroxynil and bromoxynil were readily transformed into the corresponding carboxylic acids, which formed almost 100 and 70 %, respectively, of the total product after 3 days (Table 1). The yield of carboxylic acid from ioxynil was lower in strain *R. erythropolis* A4 than in *R. rhodochrous* PA-34 (Table 1).

Acute toxicity of amides of benzonitrile herbicides

In this study, the examination of acute toxicities was carried out with the halogenated 4-hydroxybenzamides, while the corresponding nitriles and acids were tested in the previous work [32, 33]. 3,5-Dichloro-4-hydroxybenzamide, 3,5-dibromo-4-hydroxybenzamide and 3,5-diiodo-4-hydroxybenzamide caused a 50 % decrease in luminescence (EC_{50}) at concentrations of 13.9 ± 0.3, 7.9 ± 0.1 and 5.2 ± 0.03 μM, respectively. Thus, a decrease in toxicity was only observed for 3,5-dichloro-4-hydroxybenzamide compared to the corresponding nitrile (EC_{50} 5 ± 2 μM), while the brominated and iodinated benzamide analogues exhibited a higher toxicity than the

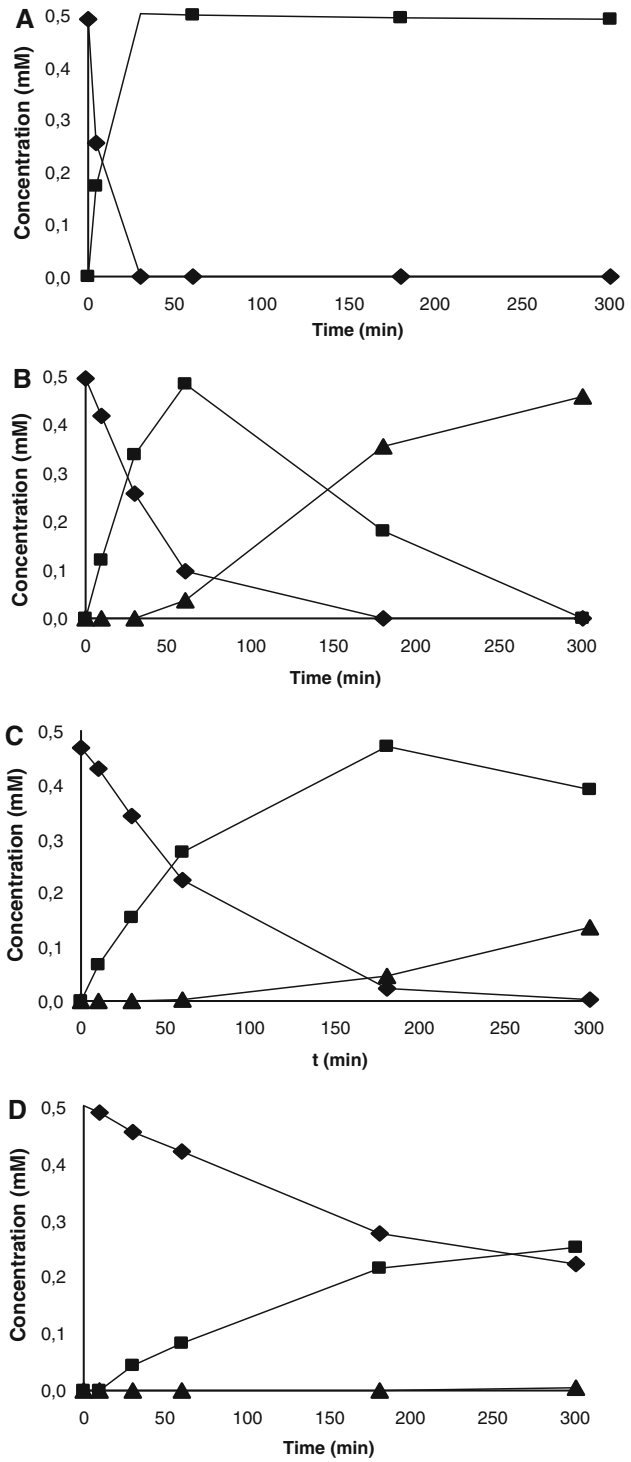

Fig. 2 Biotransformations of dichlobenil (**a**), chloroxynil (**b**), bromoxynil (**c**), and ioxynil (**d**) (0.5 mM each) by *R. erythropolis* A4. Nitriles (*diamonds*), amides (*squares*), and carboxylic acids (*triangles*). The reaction mixture (OD_{610} = 160) contained 37 U per ml of nitrile hydratase activity and 155 U per ml of amidase activity (determined for benzonitrile and benzamide, respectively)

corresponding nitriles (14 ± 3 μM and 8 ± 2 μM, respectively). 3,5-Dibromo-4-hydroxybenzoic acid with an EC_{50}-value of 42 ± 2 μM exhibited the lowest toxicity, while the EC_{50}-values determined for the other carboxylic acids (14 ± 2 μM and 6 ± 3 μM for 3,5-dichloro- and 3,5-diiodo-4-hydroxybenzoic acid, respectively) were comparable to those of the amides. Dichlobenil and its corresponding amide and acid all exhibited lower toxicities than the aforementioned compounds (approximately 505, 1,773, and 54 μM, respectively [32, 33]).

In toxicity assays using *L. sativa* seeds, the newly identified biodegradation products exerted lower inhibitory effects on germinating seeds at a concentration of 0.5 mM (53, 50, and 87 % inhibition for 3,5-dichloro-4-hydroxybenzamide, 3,5-dibromo-4-hydroxybenzamide and 3,5-diiodo-4-hydroxybenzamide, respectively) than the parent compounds (98–100 % inhibition) but similar or higher than the corresponding acids (52, 42, and 67 % inhibition, respectively) [32, 33]. Dichlobenil and the corresponding amide and acid exhibited similar or slightly higher inhibition effects (100, 93, and 60 %, respectively) at the same concentrations (*ibid.*).

Discussion

The rates of NHase-catalyzed biotransformations of benzonitrile analogues examined in this work were 3–4 orders of magnitude lower than those of benzonitrile. This could be explained by steric hindrances in the substrate molecules. In substituted 4-hydroxybenzonitriles, the electron-donating hydroxyl moiety could also play a negative role by increasing the electron density on the reactive carbon atom. In contrast, the electron-withdrawing halogen atoms exhibit a positive effect on its reactivity. This effect that became more significant with increasing electronegativity and decreasing size of the substituents (Cl > Br > I) was also reported for the nitrilase-catalyzed transformations of these compounds [32].

All tested compounds were converted by both strains, but at differing rates and with different final products. This reflected the differences in the nitrile-transforming enzymes in the strains examined. The NHase in *R. erythropolis* A4 exhibited a broad substrate specificity towards both aliphatic and aromatic nitriles and acted on bulky and sterically hindered substrates [13]. This is consistent with its ability to transform benzonitrile herbicides. There is less information available on the substrate specificity of the NHase in *R. rhodochrous* PA-34, which was reported to transform, e.g., acrylonitrile, butyronitrile, or 3-cyanopyridine [19–21].

The same nitriles and also other ones such as methacrylonitrile or chloroacetonitrile were preferentially converted by the presumably very similar L-NHase from *R. rhodochrous* J1 [36]. It can be hypothesized from the results presented for the NHase from strain PA-34, especially its very low activity for dichlobenil, that its sensitivity to steric hindrances is higher than in the *R. erythropolis* A4 enzyme.

Amidase is more sensitive to steric hindrances than NHase (e.g., [16]). In agreement with this observation, the amides produced by degradation of benzonitrile herbicides in soil were found as the main metabolites (for a review see [10]). In this work, amidase activities were much higher in *R. erythropolis* than in *R. rhodochrous*. In strains producing Fe-type NHases (*R. globerulus* A-4, *R. erythropolis* A4) or Co-type L-NHases (*R. rhodochrous* J1), the genes encoding the subunits of these enzymes are organized in clusters with genes encoding amidases [11, 37]. The latter enzymes were designated as "GGSS signature amidases" or "enantioselective amidases" [6]. In contrast, the cluster containing the gene for high molecular weight NHase in *R. rhodochrous* J1 lacks any amidase gene [11, 12]. The NHase in *R. rhodochrous* PA-34 is an L-NHase as indicated by the analysis of the purified enzyme [19] and confirmed by sequencing of parts of the genes coding for the NHase subunits in this work. Comparing the respective sequences of J1 and PA-34 enzymes, we detected only two differences within the 168 aa of the α-subunit (position 79: Ser in J1, Gly in PA-34; 101: Gly in J1, Asp in PA-34) and one difference within 204 aa of the β-subunit (position 104: Asp in J1, Glu in PA-34). The organization of the nitrile operon in the *R. rhodochrous* PA-34 strain has not yet been described and thus it is not clear if the production of its NHase is coupled to amidase synthesis. The amidase activity in the PA-34 strain may be encoded by the *amdA* homolog linked to the *nhlBA* genes as in *R. rhodochrous* J1 or by a different amidase gene located at another part of the chromosome. Rhodococci also produce amidases, which are not evolutionarily related to these enzymes but, contrary to "enantioselective amidases", they are members of branch 2 of the nitrilase superfamily [18].

Although the specific NHase activities of the rhodococci examined were relatively low for the benzonitrile herbicides, it was possible to use these strains to eliminate 0.5 mM of the herbicides from the medium, if concentrated cell suspensions were applied. Rhodococci and other strains with NHase activities were previously tested with lower dichlobenil concentrations (0.05 mM). Using both *R. erythropolis* DSM 9675 and DSM 9685, this amount of dichlobenil was removed from the medium within approximately 2 and 6 days, respectively [9]. In contrast, a tenfold higher amount was consumed within 30 min by resting cells of *R. erythropolis* A4. Using *R. erythropolis* AJ270 resting cells, 2,6-dichlorobenzamide was prepared from 60 mM dichlobenil at preparative scale with a 23 % yield after 8 days using a highly concentrated cell suspension (40 g wet weight l^{-1}).

In most of the bacteria that were reported to transform dichlobenil, 2,6-dichlorobenzamide was found as the dead-end product, but *Aminobacter* sp. also hydrolyzed 2,6-dichlorobenzamide, in addition to 2,6-dichlorobenzonitrile [23, 28]. Recent analysis of the microflora involved in 2,6-dichlorobenzamide mineralization in soils confirmed the role of *Aminobacter* sp. in this process [24]. The *Aminobacter* sp. MSHI strain seems to be promising for the bioremediation of sites contaminated with dichlobenil or its amide, being resistant to starvation conditions [25].

The rhodococci we used transformed bromoxynil and ioxynil into the corresponding amides as *Variovorax* sp. [17], but unlike this common soil organism, the rhodococcal strains, especially *R. erythropolis* A4, were also able to transform these amides further, producing carboxylic acids. Cultures of *Variovorax* sp. gave quantitative yields of amides from bromoxynil and ioxynil (0.48 μM each). Alginate-immobilized cells of *A. radiobacter* (reclassified as *R. radiobacter* [10]) also produced amides as the only products from 0.3 mM bromoxynil [31]. The absence of amides was ascribed to the steric hindrances and electrostatic potential properties of these compounds [17]. However, both the amide and acid were also previously detected by HPLC in cultures of *P. putida* able to metabolize bromoxynil in concentrations up to 0.36 mM in the presence of cosubstrates (glucose, ribose), but the acid was only found in minor amounts [34]. In the rhodococcal strains we used, the sum of the amide and acid produced was in most cases lower than the amount of the parent compound consumed, thus indicating the possibility that the acids were further transformed. The mechanism of 2,6-dichlorobenzoic acid degradation has been proposed to occur via hydroxylation and dechlorination, followed by metabolism via the *ortho*- or *meta*-pathway (for a review see [10]). Mineralization of bromoxynil was observed in soil, as well as the formation of the degradation product 3,5-dibromo-4-hydroxybenzoic acid [22] but little is known about the microbial degradation of this compound or 3,5-diiodo-4-hydroxybenzoic acid. Their aromatic rings seem to be cleaved without a prior dehalogenation [10]. Hydroxylation at the 2- and 3-positions would give rise to catechols (*ibid.*).

In conclusion, two different mechanisms of benzonitrile herbicide degradation occur in rhodococci. The first one is catalyzed by nitrilase and leads to substituted benzoic acids, while the second one consists of the hydration of the nitriles into the corresponding amides. The occurrence of this pathway in rhodococci and other soil microorganisms (*Variovorax*, *Agrobacterium*) may explain the formation of amides from benzonitrile herbicides in soils. It is probable

that the ability to transform benzonitrile herbicides is common in the nitrile-utilizing strains of *R. erythropolis* as the NHase genes are highly conserved in this species. Moreover, the enzymes are constitutive in most cases and their producers are widespread in nature. This supports the hypothesis of the important role of rhodococci in the degradation of these compounds in soils.

These organisms may also play a role in the transformation of 2,6-dichlorobenzamide and halogenated 4-hydroxybenzamides into the corresponding carboxylic acids. This study suggested that rhodococci may also be able to eliminate the carboxylic acids from the medium.

Rhodococcus erythropolis A4 and similar strains may be suitable for bioaugmentation in the removal of herbicide residues from soils. This would, however, require the metabolites to be monitored, as the conversion of the cyano group in benzonitrile herbicides into the amide or carboxy group did not lead to a significant detoxification, which seems to depend on further degradation of the intermediates.

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References

1. Avaniss-Aghajani E, Jones K, Holtzman A, Aronson T, Glover N, Boian M, Froman S, Brunk CF (1996) Molecular technique for rapid identification of mycobacteria. J Clin Microbiol 34:98–102
2. Bhalla TC, Aoshima M, Misawa S, Muramatsu R, Furuhashi K (1995) The molecular cloning and sequencing of the nitrilase gene of *Rhodococcus rhodochrous* PA-34. Acta Biotechnol 15:297–306
3. Bhalla TC, Miura A, Wakamoto A, Ohba Y, Furuhashi K (1992) Asymmetric hydrolysis of alpha aminonitriles to optically active amino acids by a nitrilase of *Rhodococcus rhodochrous* PA-34. Appl Microbiol Biotechnol 37:184–190
4. Brandão PFB, Clapp JP, Bull AT (2003) Diversity of nitrile hydratase and amidase enzyme genes in *Rhodococcus erythropolis* recovered from geographically distinct habitats. Appl Environ Microbiol 69:5754–5766
5. DiGeronimo MJ, Antoine AD (1976) Metabolism of acetonitrile and propionitrile by *Nocardia rhodochrous* LL100-21. Appl Environ Microbiol 31:900–906
6. Fournand D, Bigey F, Arnaud A (1998) Acyl transfer activity of an amidase from *Rhodococcus* sp. strain R312: formation of a wide range of hydroxamic acids. Appl Environ Microbiol 64:2844–2852
7. Freyssinet G, Pelissier B, Freyssinet M, Delon R (1996) Crops resistant to oxynils: from the laboratory to the market. Field Crops Res 45:125–133
8. Harper DB (1985) Characterization of a nitrilase from *Nocardia* sp. (*rhodochrous* group) N.C.I.B. 11215, using *p*-hydroxybenzonitrile as sole carbon source. Int J Biochem 17:677–683
9. Holtze MS, Sørensen J, Hansen HCB, Aamand J (2006) Transformation of the herbicide 2,6-dichlorobenzonitrile to the persistent metabolite 2,6-dichlorobenzamide (BAM) by soil bacteria known to harbour nitrile hydratase or nitrilase. Biodegradation 17:503–510
10. Holtze MS, Sørensen SR, Sørensen J, Aamand J (2008) Microbial degradation of the benzonitrile herbicides dichlobenil, bromoxynil and ioxynil in soil and subsurface environments—Insights into degradation pathways, persistent metabolites and involved degrader organism. Environ Pollut 154:155–168
11. Komeda H, Kobayashi M, Shimizu S (1996) A novel gene cluster including the *Rhodococcus rhodochrous* J1 *nhlBA* genes encoding a low molecular mass nitrile hydratase (L-NHase) induced by its reaction product. J Biol Chem 271:15796–15802
12. Komeda H, Kobayashi M, Shimizu S (1996) Characterization of the gene cluster of high-molecular-mass nitrile hydratase (H-NHase) induced by its reaction product in *Rhodococcus rhodochrous* J1. Proc Natl Acad Sci USA 93:4267–4272
13. Kubáč D, Kaplan O, Elišáková V, Pátek M, Vejvoda V, Slámová K, Tóthová A, Lemaire M, Gallienne E, Lutz-Wahl S, Fischer L, Kuzma M, Pelantová H, van Pelt S, Bolte J, Křen V, Martínková L (2008) Biotransformation of nitriles to amides using soluble and immobilized nitrile hydratase from *Rhodococcus erythropolis* A4. J Mol Catal B Enzym 50:107–113
14. Martínková L, Uhnáková B, Pátek M, Nešvera J, Křen V (2009) Biodegradation potential of the genus *Rhodococcus*. Environ Int 35:162–177
15. McBride KE, Kenny JW, Stalker DM (1986) Metabolism of the herbicide bromoxynil by *Klebsiella pneumoniae*. Appl Environ Microbiol 52:325–330
16. Meth-Cohn O, Wang MX (1997) An in-depth study of the biotransformation of nitriles into amides and/or acids using *Rhodococcus rhodochrous* AJ270. J Chem Soc Perkin Trans I:1099–1104
17. Nielsen MKK, Holtze MS, Svensmark B, Juhler RK (2007) Demonstrating formation of potentially persistent transformation products from the herbicides bromoxynil and ioxynil using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Pestic Manag Sci 63:141–149
18. Pace HC, Brenner C (2001) The nitrilase superfamily: classification, structure and function. Genome Biol 2:0001.1–0001.9
19. Prasad S, Raj J, Bhalla TC (2009) Purification of a hyperactive nitrile hydratase from resting cells of *Rhodococcus rhodochrous* PA-34. Ind J Microbiol 49:237–242
20. Raj J, Prasad S, Bhalla TC (2006) *Rhodococcus rhodochrous* PA-34: a potential biocatalyst for acrylamide synthesis. Process Biochem 41:1359–1363
21. Raj J, Seth A, Prasad S, Bhalla TC (2007) Bioconversion of butyronitrile to butyramide using whole cells of *Rhodococcus rhodochrous* PA-34. Appl Microbiol Biotechnol 74:535–539
22. Rosenbrock P, Munch JC, Scheunert I, Dörfler U (2004) Biodegradation of the herbicide bromoxynil and its plant cell wall bound residues in an agricultural soil. Pestic Biochem Physiol 78:49–57
23. Simonsen A, Holtze MS, Sørensen SR, Aamand J (2006) Mineralisation of 2,6-dichlorobenzamide (BAM) in dichlobenil-exposed soils and isolation of a BAM-mineralising *Aminobacter* sp. Environ Pollut 144:289–295
24. Sjøholm OR, Aamand J, Sørensen SR, Nybroe O (2010) Degrader density determines spatial variability of 2,6-dichlorobenzamide mineralization in soil. Environ Pollut 158:292–298
25. Sjøholm OR, Nybroe O, Aamand J, Sørensen SR (2010) 2,6-Dichlorobenzamide (BAM) herbicide mineralisation by *Aminobacter* sp. MSH1 during starvation depends on a subpopulation of intact cells maintaining vital membrane functions. Environ Pollut 158:3618–3625
26. Smith AE, Cullimore DR (1974) The in vitro degradation of herbicide bromoxynil. Can J Microbiol 20:773–776

27. Song L, Wang M, Shi J, Xue Z, Wang MX, Qian S (2007) High resolution X-ray molecular structure of the nitrile hydratase from *Rhodococcus erythropolis* AJ270 reveals posttranslational oxidation of two cysteines into sulfinic acids and a novel biocatalytic nitrile hydration mechanism. Biochem Biophys Res Commun 362:319–324
28. Sørensen SR, Holtze MS, Simonsen A, Aamand J (2007) Degradation and mineralization of nanomolar concentrations of the herbicide dichlobenil and its persistent metabolite 2,6-dichlorobenzamide by *Aminobacter* spp. isolated from dichlobenil-treated soils. Appl Environ Microbiol 73:399–406
29. Stalker DM, Malyj LD, McBride KE (1988) Purification and properties of a nitrilase specific for the herbicide bromoxynil and corresponding nucleotide sequence analysis of the *bxn* gene. J Biol Chem 263:6310–6314
30. Turner S, Pryer KM, Miao VP, Palmer JD (1999) Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. J Eukaryot Microbiol 46:327–338
31. Věková J, Pavlů L, Vosáhlo J, Gabriel J (1995) Degradation of bromoxynil by resting and immobilized cells of *Agrobacterium radiobacter* 8/4-strain. Biotechnol Lett 17:449–452
32. Veselá AB, Franc M, Pelantová H, Kubáč D, Vejvoda V, Šulc M, Bhalla TC, Macková M, Lovecká P, Janů P, Demnerová K, Martínková L (2010) Hydrolysis of benzonitrile herbicides by soil actinobacteria and metabolite toxicity. Biodegradation 21:761
33. Veselá AB, Franc M, Pelantová H, Kubáč D, Vejvoda V, Šulc M, Bhalla TC, Macková M, Lovecká P, Janů P, Demnerová K, Martínková L (2010) Hydrolysis of benzonitrile herbicides by soil actinobacteria and metabolite toxicity. Biodegradation 22:1255
34. Vokounová M, Vacek O, Kunc F (1992) Degradation of the herbicide bromoxynil in *Pseudomonas putida*. Folia Microbiol 37:122–127
35. Vosáhlová J, Pavlů L, Vosáhlo J, Brenner V (1997) Degradation of bromoxynil, ioxynil, dichlobenil and their mixtures by *Agrobacterium radiobacter* 8/4. Pestic Sci 49:303–306
36. Wieser M, Takeuchi K, Wada Y, Yamada H, Nagasawa T (1998) Low-molecular-mass nitrile hydratase from Rhodococcus rhodochrous J1: purification, substrate specificity and comparison with the analogous high-molecular-mass enzyme. FEMS Microbiol Lett 169:17–22
37. Xie SX, Kato Y, Komeda H, Yoshida S, Asano Y (2003) A gene cluster responsible for alkylaldoxime metabolism coexisting with nitrile hydratase and amidase in *Rhodococcus globerulus* A-4. Biochemistry 42:12056–12066