Biodegradation (2010) 21:761–770
DOI 10.1007/s10532-010-9341-4

ORIGINAL PAPER

Hydrolysis of benzonitrile herbicides by soil actinobacteria and metabolite toxicity

A. B. Veselá · M. Franc · H. Pelantová · D. Kubáč · V. Vejvoda · M. Šulc · T. C. Bhalla · M. Macková · P. Lovecká · P. Janů · K. Demnerová · L. Martínková

Received: 2 October 2009 / Accepted: 8 February 2010 / Published online: 4 March 2010

Abstract The soil actinobacteria *Rhodococcus rhodochrous* PA-34, *Rhodococcus* sp. NDB 1165 and *Nocardia globerula* NHB-2 grown in the presence of isobutyronitrile exhibited nitrilase activities towards benzonitrile (approx. 1.1–1.9 U mg^{-1} dry cell weight). The resting cell suspensions eliminated benzonitrile and the benzonitrile analogues chloroxynil (3,5-dichloro-4-hydroxybenzonitrile), bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) and ioxynil (3,5-diiodo-4-hydroxybenzonitrile) (0.5 mM each) from reaction mixtures at 30°C and pH 8.0. The products were isolated and identified as the corresponding substituted benzoic acids. The reaction rates decreased in the order benzonitrile ≫ chloroxynil > bromoxynil > ioxynil in all strains. Depending on the strain, 92–100, 70–90 and 30–51% of chloroxynil, bromoxynil and ioxynil, respectively, was hydrolyzed after 5 h. After a 20-h incubation, almost full conversion of chloroxynil and bromoxynil was observed in all strains, while only about 60% of the added ioxynil was converted into carboxylic acid. The product of ioxynil was not metabolized any further, and those of the other two herbicides very slowly. None of the nitrilase-producing strains hydrolyzed dichlobenil (2,6-dichlorobenzonitrile). 3,5-Dibromo-4-hydroxybenzoic acid exhibited less inhibitory effect than bromoxynil both on luminescent bacteria and germinating seeds of *Lactuca sativa*. 3,5-Diiodo-4-hydroxybenzoic acid only exhibited lower toxicity than ioxynil in the latter test.

Keywords Nitrilase · Benzonitrile herbicides · Chloroxynil · Bromoxynil · Ioxynil · Actinobacteria

A. B. Veselá · M. Franc · D. Kubáč · V. Vejvoda · L. Martínková (✉)
Laboratory of Biotransformation, Institute of Microbiology, Academy of Sciences of the Czech Republic (ASCR), Vídeňská 1083, 142 20 Praha, Czech Republic
e-mail: martinko@biomed.cas.cz

H. Pelantová · M. Šulc
Laboratory of Molecular Structure Characterization, Institute of Microbiology, Academy of Sciences of the Czech Republic (ASCR), Vídeňská 1083, 142 20 Praha, Czech Republic

H. Pelantová
Department of Analytical Chemistry, Faculty of Science, Palacký University, Tř. Svobody 8, 771 46 Olomouc, Czech Republic

T. C. Bhalla
Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla 171005, India

M. Macková · P. Lovecká · P. Janů · K. Demnerová
Faculty of Food and Biochemical Technology, Institute of Chemical Technology Prague, Technická 5, 166 28 Prague, Czech Republic

Introduction

Dihalogenated benzonitrile analogues are active compounds in a number of herbicides. The use, chemical and physical properties, toxicity, degradation and

metabolites of this herbicide group have been reviewed recently (Holtze et al. 2008). The broad-spectrum contact herbicide dichlobenil (2,6-dichlorobenzonitrile) is used to control weeds in gardens, orchards, plant nurseries, on non-agricultural areas etc. Bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) and ioxynil (3,5-diiodo-4-hydroxybenzonitrile) are contact herbicides selective for broad-leaved weeds among cereal crops. They are consumed in higher quantities than dichlobenil due to their application to larger areas. The risk of degradation of the benzonitrile herbicides into persistent metabolites has raised some concerns. Indeed, the major metabolite of dichlobenil is 2,6-dichlorobenzamide, which is hardly biodegradable and more soluble than the parent compound. In addition, its sorption affinity in soil and subsurface sediments is much lower. As a result, it has been frequently found in groundwater. Biodegradation studies also detected amides as the major products of bromoxynil and ioxynil. These metabolites are also more soluble than the original compounds, and presumably possess a medium mobility in soils. Their toxicity and environmental dissipation remain largely unknown.

The processes underlying the biodegradation of benzonitrile herbicides in soils are not yet well understood. However, experiments with pure bacterial cultures indicate that these compounds are attacked at their nitrile group. The substituent position in their molecules strongly affects their biodegradability by nitrile-trasforming enzymes. Thus dichlobenil is a substrate of a number of nitrile hydratases (for a review, see Holtze et al. 2008), but apparently no nitrilase. *Rhizobium radiobacter* (formerly *Agrobacterium radiobacter*; Vosáhlová et al. 1997; Holtze et al. 2006) and *Variovorax* sp. (Nielsen et al. 2007) not only converted dichlobenil, but also bromoxynil and ioxynil into the corresponding amides. Alternatively, bromoxynil and ioxynil were hydrolyzed into carboxylic acids by the nitrilase of *Klebsiella pneumoniae* ssp. *ozaenae* (McBride et al. 1986; Stalker et al. 1988) and bromoxynil by *Pseudomonas putida* (Golovleva et al. 1988). The corresponding gene from *Klebsiella* was utilized for constructing transgenic plants with bromoxynil and ioxynil resistance (Freyssinet et al. 1996). A marginal note on very low relative activities towards bromoxynil and ioxynil appeared in the study on the purification and characterization of nitrilase from *Fusarium solani* by (Harper 1977). A strain of *Klebsiella* genus (*K. ozaenae*) and a *F. solani* strain were also able to degrade ioxynil into CO_2 (Hsu and Camper 1976).

Actinobacteria, primarily the genus *Rhodococcus*, were found to be efficient in degrading of a large number of environmental contaminants, including various nitriles (Martínková et al. 2009). However, there have been few reports on their ability to degrade benzonitrile herbicides. Several strains of rhodococci, specifically *R. erythropolis* DSM 9675, DSM 9685 (Holtze et al. 2006) and AJ270 (Blakey et al. 1995; Meth-Cohn and Wang 1997) harbouring nitrile hydratases were shown to transform dichlobenil into 2,6-dichlorobenzamide as the dead-end product. A very low activity towards bromoxynil and ioxynil was demonstrated for the purified nitrilase from *Rhodococcus rhodochrous* NCIB 11215 (previously classified as *Nocardia* sp.; Harper 1985). This enzyme transformed bromoxynil and ioxynil at a V_{max} of 1.0 and 1.6% of that for benzonitrile, respectively (Harper 1985). Furthermore, the K_m-values of this enzyme for bromoxynil and ioxynil were much higher than that for benzonitrile (8.18, 11.88 and 0.62 mM, respectively). McBride et al. (1986) confirmed the very low nitrilase activity for bromoxynil in this organism (less than 0.3% of that for benzonitrile), while using this strain for comparison with the bromoxynil-degrading strain *Klebsiella pneumoniae* ssp. *ozaenae.*

The aim of this work was to examine the potential role of actinobacteria, as common soil-dwelling organisms, in the degradation of benzonitrile herbicides. We focused this study on nitrilase-producing strains whose ability to degrade these compounds has been largely unknown. We therefore chose the soil isolates *Rhodococcus rhodochrous* PA-34 (Bhalla et al. 1992; Raj et al. 2006, 2007), *Nocardia globerula* NHB-2 (Bhalla and Kumar 2005) and *Rhodococcus* sp. NDB 1165 (Prasad et al. 2007), which were all previously shown to produce nitrilases acting on (hetero)aromatic nitriles. Having confirmed carboxylic acids to be major products of the herbicide transformation in these strains, we determined the acute toxicities of these metabolites and the parent compounds by using luminescent bacteria and germinating seeds.

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—benzamide, benzoic acid, 3,5-dichloro-4-hydroxybenzoic acid, 3,5-dibromo-4-hydroxybenzoic acid, 3,5-diiodo-4-hydroxybenzoic acid, 2,6-dichlorobenzamide and 2,6-dichlorobenzoic acid—were purchased from standard commercial sources (Sigma-Aldrich; Alfa Aesar, Germany) and were of analytical grade purity.

Microorganisms

Rhodococcus rhodochrous PA-34 (Bhalla et al. 1992), *Nocardia globerula* NHB-2 (Bhalla and Kumar 2005; previously classified as *Rhodococcus rhodochrous*, Sankhian et al. 2003) and *Rhodococcus* sp. NDB 1165 (Prasad et al. 2007) were as described previously. All strains were maintained on meat peptone agar: [g/l] Bacto beef extract 3, peptone 10, NaCl 5, agar 15 g (pH 7.5), and periodically transferred to fresh media. Strains were grown in 500-ml Erlenmeyer flasks with 100 ml of a mineral medium (pH 7.5) containing: [g/l] $Na_2HPO_4 \cdot 12H_2O$ 2.5, KH_2PO_4 2.0, $MgSO_4 \cdot 7H_2O$ 0.5, $FeSO_4 \cdot 7H_2O$ 0.03, $CaCl_2 \cdot 2H_2O$ 0.06, yeast extract 0.1, glucose 10, isobutyronitrile 3.85 (55 mM). This medium was inoculated with 8% (v/v) of a preculture grown for 1 day at 30°C in a medium containing: [g/l] Bacto beef extract 10, peptone 10, NaCl 5, glucose 10, yeast extract 1 (pH 7.5). From cultures grown for 1 day under shaking (200 rpm) at 28°C, cells were harvested by centrifugation, washed with and resuspended in Tris/HCl buffer (50 mM, pH 8.0) and immediately used for enzyme activity assays and biodegradation studies. Alternatively, chloroxynil, bromoxynil or ioxynil (0.5 mM each) were added to the culture medium instead of isobutyronitrile and the cultivation was performed for 6 days under the above conditions.

Enzyme assays

For determining nitrilase activity, whole-cell suspensions (475 µl; optical density (OD_{610}) ≅ 1 corresponding to approx. 0.28 mg of dry cell weight/ml) were preincubated at 30°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 30°C for 10 min, the reactions were stopped by adding 100 µl of 1 M HCl. The reactions mixtures were centrifuged (14000 rpm, 5 min) and analyzed for reaction product (benzoic acid) by HPLC. One unit of nitrilase activity was defined as the amount of enzyme that formed 1 µmol of benzoic acid per min under the above conditions. For determination of amidase activity, whole-cell suspensions (585 µl; OD_{610} ≅ 1) were preincubated at 30 or 45°C for 5 min and benzamide (15 µl of 100 mM stock solution in methanol) was added to a final concentration of 2.5 mM. After shaking at 850 rpm and 30 or 45°C for 10 min, the reactions were stopped by adding 100 µl of 1 M HCl. The reaction mixtures were centrifuged (14000 rpm, 5 min) and analyzed for the reaction product (benzoic acid) by HPLC. One unit of amidase activity was defined as the amount of enzyme that formed 1 µmol of benzoic acid per min under the above conditions.

Biotransformations

In analytical scale experiments, the biotransformations were carried out at 30°C and shaking (850 rpm) in 1.5-ml eppendorf tubes with 1200 µl of the reaction mixtures consisting of whole-cell suspensions (1140 µl; OD_{610} ≅ 2) and the substrates (chloroxynil, bromoxynil, ioxynil or dichlobenil; 60 µl from 10 mM stock solutions in methanol; final concentration 0.5 mM). Control abiotic experiments with the benzonitrile analogues and the corresponding carboxylic acids (0.5 mM each) were performed under the same conditions in buffer without cells. Whole cells from precultures were used for control experiments with uninduced cells.

In preparative scale experiments, the biotransformations were carried out at 30°C and shaking (200 rpm) in 250-ml Erlenmeyer flasks with 50 ml of the reaction mixtures. Chloroxynil, bromoxynil or ioxynil (0.1 mmol each) were dissolved in 2.5 ml of methanol and 47.5 ml of the cell suspensions (OD_{610} ≅ 2) were added.

The biotransformation of benzonitrile analogues were monitored by TLC on silica gel 60 GF254 (Merck) plates developed in dichloromethane-methanol-25% aqueous ammonia (15:5:1).

Analytical HPLC

Samples (150 µl) from the reactions were mixed with 150 µl of methanol, centrifuged, appropriately diluted with a mobile phase consisting of acetonitrile:water:H_3PO_4, 250:749:1, and analyzed for nitriles, carboxylic acids and amides using a Waters HPLC system (Waters 600 pump and Waters PDA 996 detector) equipped with a Chromolith Flash RP-18 Merck monolithic column (25 mm × 4.6 mm) at a flow rate of 2 ml min^{-1} and 35°C (see Table 1 for retention times and local spectral maxima of the analytes).

Product isolation and identification

The retention times and UV spectra (Table 1) were compared with those of authenthic standards. To isolate the products, the reaction mixtures from preparative biotransformations were centrifuged (6000 rpm, 30 min, 4°C) and the pH of the supernatants adjusted to 2–2.5 using 1 M HCl. The product was extracted three times with 50 ml of ethyl acetate. The combined organic layers were dried with Na_2SO_4 and the solvent removed by evaporation under reduced pressure. NMR spectra were recorded with a Bruker Avance III spectrometer (400.13 MHz for ^1H, 100.61 MHz for ^{13}C, DMSO-d_6, 30°C) using Topspin 2.1 software. Residual solvent signal (DMSO: δ_H 2.50 ppm, δ_C 39.60 ppm) was used as an internal standard.

Mass spectrometry analysis and MS/MS experiments were performed using a LCQ^{DECA} ion trap mass spectrometer (ThermoQuest, San Jose, CA) equipped with a static nanoelectrospray ion source. The spray voltage was held at 1.42 kV, the tube lens voltage was −10 V. The heated capillary was kept at 180°C with a voltage of −37 V. Negative-ion full scans were acquired over the *m/z* range 150–2000. To corroborate the structure of metabolites the MS/MS experiments were performed with normalised collision energy in range 30–45%, activation Q 0.25, activation time 30 ms and with isolation width *m/z* 3.0.

Spectral data are only shown for products prepared using the *R. rhodochrous* PA-34 strain. The data obtained for products of other strains indicated the same structures.

3,5-Dichloro-4-hydroxybenzoic acid (**1b**): ^1H NMR: 7.824 (2H, s, H-2, H-6); ^{13}C NMR: 122.08 (C-3, C-5), 123.36 (C-1), 129.70 (C-2, C-6), 153.19 (C-4), 165.24 (CO). Negative MS (nESI): *m/z* 205.1, 207.0 ($C_7H_4Cl_2O_3$).

3,5-Dibromo-4-hydroxybenzoic acid (**2b**): ^1H NMR: 7.999 (2H, s, H-2, H-6); ^{13}C NMR: 111.42 (C-3, C-5), 124.62 (C-1), 133.45 (C-2, C-6), 154.94 (C-4), 164.99 (CO). Negative MS (nESI): *m/z* 293.1, 295.0, 297.0 ($C_7H_4Br_2O_3$).

3,5-Diiodo-4-hydroxybenzoic acid (**3b**): ^1H NMR: 8.212 (2H, s, H-2, H-6); ^{13}C NMR: 85.91 (C-3, C-5), 126.12 (C-1), 140.46 (C-2, C-6), 159.57 (C-4), 164.65 (CO). Negative MS (nESI): *m/z* 388.9 ($C_7H_4I_2O_3$).

Table 1 Analytical HPLC of benzonitrile, benzonitrile analogues and products of their biotransformation (see "Materials and methods" section for separation conditions)

Compound	Retention time [min]	Local spectral maximum [nm]
3,5-Dichloro-4-hydroxybenzonitrile (**1a**)	1.6	215.8[a]–255.8
3,5-Dichloro-4-hydroxybenzoic acid (**1b**)	0.8	215.8[a]–251
3,5-Dibromo-4-hydroxybenzonitrile (**2a**)	2.3	216.9[a]–256.9
3,5-Dibromo-4-hydroxybenzoic acid (**2b**)	1.0	216.9[a]–256.9
3,5-Diiodo-4-hydroxybenzonitrile (**3a**)	4.0	235.7[a]
3,5-Diiodo-4-hydroxybenzoic acid (**3b**)	1.5	235.7[a]
2,6-Dichlorobenzonitrile	4.0	–[b]
2,6-Dichlorobenzamide	0.5	–[b]
2,6-Dichlorobenzoic acid	0.8	–[b]
Benzonitrile	1.3	222.8[a]
Benzamide	0.5	225.2[a]
Benzoic acid	0.8	228.7[a]

[a] wavelength used for compound detection and quantitation

[b] detected and quantified at 210 nm

Toxicity test using *Vibrio fischeri*

Lyophilised cells of *Vibrio fischeri* were resuscitated in 2% NaCl at 15°C. The cell suspensions were incubated with the examined compounds for 15 min at 15°C and luminescence was determined using a Lumac Biocounter 1500 at the beginning of incubation (about 6000 relative light units) and at its end. The EC_{50} value was defined as the compound concentration at which luminescence was inhibited by 50%, and was calculated from four measurements carried out for each of four concentrations of the examined compounds.

Toxicity test using *Lactuca sativa*

Germinating *Lactuca sativa* seeds were incubated with 0.5 mM of the examined compounds at 22°C in dark and the root length was determined after 4 days. The inhibition coefficient I was defined as

$$I = \frac{L_C - L_T}{L_C} \cdot 100,$$

where L_c is the average root length in seeds germinating in reference solution (cm), and L_T is average root length in seeds germinating in test solutions (cm).

The reference solution contained (in mg/l of distilled water) $CaCl_2 \cdot 2H_2O$ 294, $MgSO_4 \cdot 7H_2O$ 123, $NaHCO_3$ 65, KCl 6.

A statistically significant difference between the average root length of seeds germinating in reference solution and test solutions was determined using the ANOVA test with a significance level of 95%.

Results

Nitrile- and amide-transforming activities of actinobacteria

All three strains produced nitrilase activities, when grown in the presence of isobutyronitrile as nitrilase inducer (Table 2). Benzoic acid was formed as the single detectable product from benzonitrile in all strains. This suggested that benzonitrile hydrolysis was catalyzed by the nitrilase under the conditions used. No production of benzamide indicated no nitrile hydratase activity in the cells. To confirm that benzamide was not removed by the action of an amidase, the cells were examined for this activity at 45°C (largely suitable for rhodococcal amidases; Banerjee et al. 2002) and 30°C (to detect potential thermosensitive amidases). At both 45°C (Table 2) and 30°C (*data not shown*), the amidase activities towards benzamide were comparable and two orders of magnitude lower than the nitrilase activities in all strains. Therefore, benzamide, when formed by nitrile hydratase, would largely remain unreacted in the reaction mixtures.

A comparison of the specific activities for benzonitrile and benzonitrile herbicides was difficult to perform, as it was impossible to carry out the activity assays under the same reaction conditions for all these substrates. The concentrations of products formed by whole cells from benzonitrile analogues were too low after 10-min reactions, which were used for the activity assays with benzonitrile. The determination of activity for benzonitrile herbicides was also hampered by the very low solubility of these compounds in water (approx. 0.47 and 0.13 mM for bromoxynil and ioxynil, respectively, at 20°C; Holtze et al. 2008). Nevertheless, the analysis of samples obtained after 30-min reactions with e.g., *N. globerula* NHB-2, suggested nitrilase activities towards chloroxynil, bromoxynil and ioxynil (approx. 7.3, 4.0 and 1.4 mU mg^{-1} dry cell weight, respectively) to be two to three orders of magnitude lower in comparison to those towards benzonitrile (Table 2). In the other two strains, the nitrilase activities for chloroxynil, bromoxynil and ioxynil were somewhat higher in most cases (strain PA-34: 14.6, 8.8 and 0.6 mU mg^{-1}, respectively; strain NDB 1165: 16.1, 10.3 and 3.8 mU mg^{-1}, respectively) compared to strain NHB-2, but also much lower in comparison with the activities for benzonitrile (Table 2). None of the strains showed any nitrilase activity for dichlobenil.

The cultures grown in presence of chloroxynil, bromoxynil or ioxynil (0.5 mM each) instead of isobutyronitrile were not able to transform any of the benzonitrile herbicides, the concentrations of which remained almost unchanged in the culture medium within 6 days of cultivation. This indicated that no nitrilase activity was induced under these conditions.

Products of benzonitrile herbicide biodegradation

Despite the low activities of the nitrilase-producing strains for benzonitrile herbicides, the concentrations of these compounds, except for dichlobenil, decreased significantly during incubation with whole-cell suspensions of these organisms grown on isobutyronitrile

Table 2 Nitrilase and amidase activities of various actinobacteria (see "Materials and methods" section for details)

Strain	Nitrilase activity		Amidase activity	
	Specific (U/mg)[a]	Total (U/l)[b]	Specific (U/mg)[a]	Total (U/l)[b]
Rhodococcus rhodochrous PA-34	1.41	489	0.014	4.9
Rhodococcus sp. NDB 1165	1.08	292	0.004	1.09
Nocardia globerula NHB-2	1.87	564	0.011	3.3

[a] per mg of dry cell weight

[b] per L of culture broth

as nitrilase inducer. Contrary, almost no change in concentration of any of the compounds was observed in abiotic experiments or those using uninduced cells (within 5 h). In biotransformations of chloroxynil (**1a**), bromoxynil (**2a**) and ioxynil (**3a**), HPLC analysis revealed the formation of products exhibiting the same retention times and UV spectra as the corresponding substituted benzoic acids **1**–**3b**. To obtain the products for the spectroscopical characterization, the nitriles were incubated with resting cell suspensions of each strain at a preparative scale. The products were isolated and spectroscopically characterized by NMR and MS as the expected substituted benzoic acids in all cases (Table 1; see Materials and methods for spectral data). Comparison of the chromatograms of the reaction mixtures with dichlobenil as the substrate with those of authentic standards of 2,6-dichlorobenzoic acid and 2,6-dichlorobenzamide did not show any formation of these compounds by any of the strains tested.

Biodegradation rates

After the identification of major degradation products as the substituted benzoic acids **1**–**3b**, their peaks in HPLC were calibrated using authentic standards and their concentrations in the reaction mixtures with whole cells monitored over time (Fig. 1). Chloroxynil and bromoxynil were converted into almost stoichiometric amounts of the corresponding carboxylic acids, indicating no significant formation of other products within the monitored period of time (5 h). Minor deviations from the expected sum of substrate and product were observed throughout the reactions with bromoxynil (approx. 10%), probably because of its limited solubility (0.47 mM). The stoichiometry of ioxynil hydrolysis could not be assessed due to a very low solubility of the substrate (0.13 mM).

Chloroxynil was transformed into almost equimolar amounts of the corresponding carboxylic acid within 5 h and bromoxynil was hydrolyzed by 70–90%. The transformation of ioxynil proceeded more slowly, 30–51% of the substrate being hydrolyzed after 5 h. The reaction rate decrease in the order chloroxynil > bromoxynil > ioxynil probably reflected both steric and electronic effects in the substrate molecules (increasing bulkiness and decreasing electron-withdrawing effect of halogen atoms).

After a 20-h incubation, almost total conversion of chloroxynil and bromoxynil was achieved with all strains. On the other hand, ioxynil was not fully converted by any of the strains even after a 3-day incubation, the highest conversion being about 60% in all strains (*data not shown*).

The carboxylic acid formed from ioxynil was not metabolized further by the whole cells to a significant extent. On the other hand, some decrease in the concentrations of the carboxylic acids obtained from the other nitriles occurred between day 1 and 3 of incubation (*data not shown*). The maximum decrease in 3,5-dichloro-4-hydroxybenzoic acid and 3,5-dibromo-4-hydroxybenzoic acid was by about 25 and 18% during this time in the reaction mixture with cells of *R. rhodochrous* PA-34 and *N. globerula*, respectively. No peaks of potential breakdown intermediates were found in the chromatograms in any experiment. There was no significant decrease in concentrations of these compounds in abiotic controls.

Acute toxicity of benzonitrile herbicides and their biodegradation products

In chemiluminescence inhibition tests (Table 3), bromoxynil was found to be considerably more toxic than the corresponding carboxylic acid. The toxicities of ioxynil and its acid were similar and the highest of

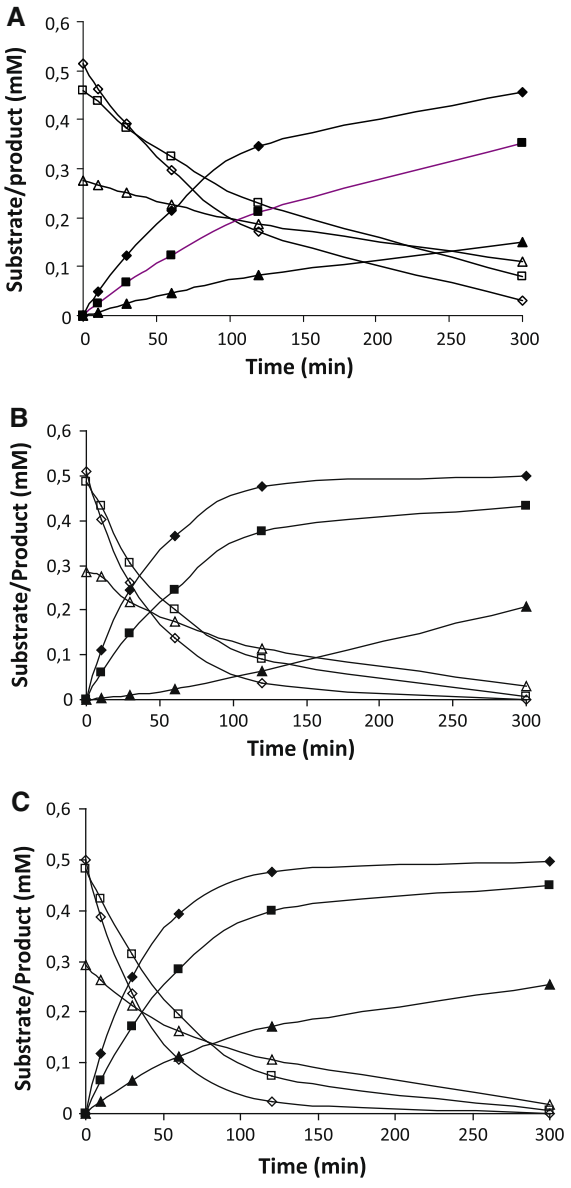

Fig. 1 Rates of nitrile consumption (**1**–**3a**; *open symbols*) and carboxylic acid (**1**–**3b**; *filled symbols*) formation in biotransformations of chloroxynil (*diamond*), bromoxynil (*square*) and ioxynil (*triangle*) by *Nocardia globerula* NHB-2 (**a**), *Rhodococcus rhodochrous* PA-34 (**b**) and *Rhodococcus* sp. NDB 1165 (**c**). Optical density of reaction mixtures was approx. 2 in all cases. See "Materials and methods" section for details

all the compounds. Dichlobenil and its corresponding amide exhibited a much lower toxicity than all the hydroxylated compounds. The toxicity of 2,6-dichlorobenzoic acid, however, was comparable to that of 3,5-dibromo-4-hydroxybenzoic acid. In toxicity assays using *Lactuca sativa* seeds (Table 4), all of the benzonitrile analogues tested at 0.5 mM caused an almost complete inhibition of root growth. At these concentrations, all the biodegradation products exerted less inhibitory effect on the plants than the parent compounds. However, at concentrations of 0.05 mM, 3,5-dichloro-4-hydroxybenzoic acid and 3,5-dibromo-4-hydroxybenzoic acid exhibited a similar inhibitory activity as the corresponding nitriles.

Table 3 Determination of acute toxicity of chloroxynil, bromoxynil, ioxynil, dichlobenil and standards of their biodegradation products using the luminescent bacterium *Vibrio fischeri*

Compound	EC_{50} ± SD (nM)
3,5-Dichloro-4-hydroxybenzonitrile	5 ± 2
3,5-Dichloro-4-hydroxybenzoic acid	14 ± 2
3,5-Dibromo-4-hydroxybenzonitrile	14 ± 3
3,5-Dibromo-4-hydroxybenzoic acid	42 ± 2
3,5-Diiodo-4-hydroxybenzonitrile	8 ± 2
3,5-Diiodo-4-hydroxybenzoic acid	6 ± 3
2,6-Dichlorobenzonitrile	505 ± 29
2,6-Dichlorobenzamide	1773 ± 53
2,6-Dichlorobenzoic acid	54 ± 11

EC_{50} compound concentration causing 50% inhibition of luminescence, *SD* standard deviation

The toxicity assay was performed as described in "Materials and methods" section

Table 4 Determination of the effect of chloroxynil, bromoxynil, ioxynil, dichlobenil and standards of their biodegradation products on root growth of germinating seeds of *Lactuca sativa*

Compound	Inhibition (%)	
	0.05 mM	0.5 mM
3,5-Dichloro-4-hydroxybenzonitrile	32	100
3,5-Dichloro-4-hydroxybenzoic acid	30	52
3,5-Dibromo-4-hydroxybenzonitrile	46	98
3,5-Dibromo-4-hydroxybenzoic acid	42	42
3,5-Diiodo-4-hydroxybenzonitrile	64	100
3,5-Diiodo-4-hydroxybenzoic acid	41	67
2,6-Dichlorobenzonitrile	100	100
2,6-Dichlorobenzamide	11	93
2,6-Dichlorobenzoic acid	45	60

The toxicity assay was performed as described in "Materials and methods" section

Discussion

The *Rhodococcus rhodochrous* PA-34, *Nocardia globerula* NHB-2 and *Rhodococcus* sp. NDB 1165 strains have been previously shown to produce nitrilases with high relative activities for benzonitrile (Bhalla et al. 1992, Bhalla and Kumar 2005, Prasad et al. 2007) or 3- and 4-cyanopyridine (Prasad et al. 2007). These enzymes can be, therefore, designated as aromatic nitrilases according to the classification of Kobayashi and Shimizu (1994). Chloroxynil, bromoxynil and ioxynil were transformed by all the enzymes tested, though with low relative activities compared to benzonitrile (presumably due to steric hindrances).

Previously, only one rhodococcal nitrilase was reported to be active for bromoxynil and ioxynil (Harper 1985; McBride et al. 1986). This enzyme was obtained from the *R. rhodochrous* NCIMB11215 strain (originally characterized as *Nocardia* sp. (*rhodochrous* group)) isolated from a bromoxynil-treated field. However, apart from ammonia, the products of the reactions were not determined. In contrast, in this work the major biodegradation products were identified (as the corresponding carboxylic acids).

The nitrilases from the *R. rhodochrous* PA-34 and *R. rhodochrous* NCIMB11215 strains seemed to be different because of their differing specific activities for benzonitrile (24 and 1.74 U mg^{-1}) (Bhalla et al. 1992; Harper 1985). Their substrate specificities could hardly be compared, as different substrates were used for their characterization (Harper 1985; Bhalla et al. 1992). The nitrilase from *R. rhodochrous* PA-34 was shown to be highly similar in terms of its amino acid sequence with the well-characterized nitrilase from *R. rhodochrous* J1 (with 97.5% homology) but to share only a 44.6% homology with the benzonitrile herbicide-specific nitrilase from *Klebsiella pneumoniae* ssp. *ozaenae* (Bhalla et al. 1995). This latter enzyme strikingly differs from rhodococcal nitrilases in its substrate specificity, exhibiting higher specific activities and lower K_m-values for chloroxynil, bromoxynil and ioxynil (V_{max} of 18, 15 and 12.2 U mg^{-1} protein and K_m of 0.83, 0.31 and 0.55 mM, respectively) but no detectable activity for benzonitrile (Stalker et al. 1988).

The biodegradation potential of *K. pneumoniae* ssp. *ozaenae* was demonstrated in a growing culture, which consumed almost 1.8 mM of bromoxynil from the culture medium within 18 h (McBride et al. 1986). Analogous experiments with cultures of actinobacteria, growing, however, at lower concentrations (0.5 mM) of the benzonitrile herbicides, did not result in any degradation of these compounds. In contrast, it was necessary to induce the nitrilase activity in the cells by growth in presence of isobutyronitrile, a powerful nitrilase inducer (Bhalla et al. 1992), prior to the biodegradation experiments. However, it is difficult to assess from these observations, if and to which extent these actinobacteria participate in degradation of benzonitrile herbicides in soil, as different conditions in this environment (e.g., lower herbicide concentrations, potential presence of other nitrilase inducers, longer exposure to the herbicides) must be considered.

A considerable amount of data has been obtained regarding the toxicities of benzonitrile herbicides (Holtze et al. 2008; EC 2004a,b; http://extoxnet.orst.edu), but much less has been known on those of their biodegradation products. The difference in the toxicity of dichlobenil and its amide on one hand and bromoxynil and ioxynil on the other hand, as determined by the chemiluminescence test, is in accordance with the LD_{50} values of these compounds (Holtze et al. 2008). The low relative toxicity of 3,5-dibromo-4-hydroxybenzoic acid was demonstrated by a report that this metabolite caused liver and developmental effects at higher dose levels than bromoxynil (EC 2004a). The results of the chemiluminescence test also suggested a lower toxicity for this metabolite compared to the parent compound. The nitriles inhibited root growth in *L. sativa* the most but significant inhibitory effect was observable with all the derivatives (amide, carboxylic acids). A residual (20-fold lower) inhibitory activity was also reported for 3,5-dihalogeno-4-hydroxybenzoic acids by Carpenter and Heywood (1963).

Conclusions

We have shown that all three tested strains of soil actinobacteria isolated from different sites were able to hydrolyze the benzonitrile analogues chloroxynil, bromoxynil and ioxynil. We have determined the nitrilase to be the key enzyme in this pathway. Previously, the direct hydrolysis of benzonitrile herbicides had been considered to be the ability of

specific nitrilases occurring in the *Klebsiella* genus. However, the frequency of occurrence of bromoxynil and ioxynil degraders in this genus is unknown, as the corresponding studies were largely concerned with a single strain. Our findings on nitrilase activity for these herbicides in randomly selected strains of *Rhodococcus* and *Nocardia* genera suggest that this ability is not specific to genus *Klebsiella*, and probably widespread in actinobacteria. Halogenated benzoic acids were confirmed as the products of biotransformations catalyzed by actinobacteria. These strains may also be used to convert bromoxynil into a less toxic metabolite—3,5-dibromo-4-hydroxybenzoic acid, which, moreover, may be further utilized by consortia of soil organisms. However, the impact of bioaugmentation with the nitrile degraders on metabolite profiles must be studied to assess the utility of these strains in bioremediation of sites contaminated with benzonitrile herbicides.

Acknowledgments Financial support via projects FT-TA5/043 (Ministry of Industry and Commerce of the Czech Republic), OC09046, LC06010 (Ministry of Education of the Czech Republic), IAA500200708 (Grant Agency of the Academy of Sciences of the Czech Republic), 305/09/H008 (Czech Science Foundation) and the institutional research concept AV0Z50200510 (Institute of Microbiology) are gratefully acknowledged.

References

Banerjee A, Sharma R, Banerjee UC (2002) The nitrile-degrading enzymes: current status and future prospects. Appl Microbiol Biotechnol 60:33–44. doi:10.1007/s00253-002-1062-0

Bhalla TC, Kumar H (2005) *Nocardia globerula* NHB-2: a versatile nitrile-degrading organism. Can J Microbiol 51(8):705–708. doi:10.1139/w05-046

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(2): 184–190

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(3):297–306. doi:10.1002/abio.370150308

Blakey AJ, Colby J, Williams E, O'Reilly C (1995) Regio- and stereo-specific nitrile hydrolysis by the nitrile hydratase from *Rhodococcus* AJ270. FEMS Microbiol Lett 129(1): 57–62

Carpenter K, Heywood BJ (1963) 3:5-Dihalogeno-4-hydroxybenzonitriles as herbicides: Herbicidal action of 3:5-Dihalogeno-4-hydroxybenzonitriles. Nature 200:28–29

EC (2004a) Review report for the active substance bromoxynil. European Commission, SANCO/4347/2000. http://ec.europa.eu/food/plant/protection/evaluation/existactive/list_bromoxynil.pdf

EC (2004b) Review report for the active substance ioxynil. European Commission, SANCO/4349/2000. http://ec.europa.eu/food/plant/protection/evaluation/existactive/list_ioxynil.pdf

Freyssinet G, Pelissier B, Freyssinet M, Delon R (1996) Crops resistant to oxynils: from the laboratory to the market. Field Crops Res 45(1–3):125–133

Golovleva LA, Pertsova RN, Kunc F, Vokounová M (1988) Decomposition of the herbicide bromoxynil in soil and in bacterial cultures. Folia Microbiol 33(6):491–499. doi: 10.1007/BF02925776

Harper DB (1977) Fungal degradation of aromatic nitriles. Enzymology of C–N cleavage by *Fusarium solani*. Biochem J 167(3):685–692

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(6):677–683

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(6):503–510. doi:10.1007/s10532-005-9021-y

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(2):155–158. doi:10.1016/j.envpol.2007.09.020

Hsu JC, Camper ND (1976) Isolation of ioxynil degraders from soil-enrichment cultures. Can J Microbiol 22(4):537–543. doi:10.1139/m76-080

Kobayashi M, Shimizu S (1994) Versatile nitrilases: Nitrile-hydrolysing enzymes. FEMS Microbiol Lett 120(3):217–224. doi:10.1016/0378-1097(94)90474-X

Martínková L, Uhnáková B, Pátek M, Nešvera J, Křen V (2009) Biodegradation potential of the genus *Rhodococcus*. Environ Int 35:162–177. doi:10.1016/j.envint.2008.07.018

McBride KE, Kenny JW, Stalker DM (1986) Metabolism of the herbicide bromoxynil by *Klebsiella pneumoniae* subsp. *ozaenae*. Appl Environ Microbiol 52(2):325–330

Meth-Cohn O, Wang M-X (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(8):1099–1104

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). Pest Manag Sci 63:141–149. doi: 10.1002/ps.1311

Prasad S, Misra A, Jangir VP, Awasthi A, Raj J, Bhalla TC (2007) A propionitrile-induced nitrilase of *Rhodococcus* sp. NDB 1165 and its application in nicotinic acid synthesis. World J Microbiol Biotechnol 23(3):345–353. doi: 10.1007/s11274-006-9230-5

Raj J, Prasad S, Bhalla TC (2006) *Rhodococcus rhodochrous* PA-34: a potential biocatalyst for acrylamide synthesis. Proc Biochem 41(6):1359–1363. doi:10.1016/j.procbio.2006.01.022

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(3):535–539. doi:10.1007/s00253-006-0693-y

Sankhian UD, Kumar H, Chand D, Kumar D, Bhalla TC (2003) Nitrile hydratase of *Rhodococcus rhodochrous* NHB-2: optimisation of conditions for production of enzyme and conversion of acrylonitrile to acrylamide. Asian J Microbiol Biotechnol Environ Sci 5(2):217–223

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(13):6310–6314

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(3):303–306. doi:10.1002/(SICI)1096-9063(199703)49:3<303:AID-PS519>3.0.CO;2-T