JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Biotransformation of the Neonicotinoid Insecticide Thiacloprid by the Bacterium *Variovorax boronicumulans* Strain J1 and Mediation of the Major Metabolic Pathway by Nitrile Hydratase

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ABSTRACT: A neonicotinoid insecticide thiacloprid-degrading bacterium strain J1 was isolated from soil and identified as *Variovorax boronicumulans* by 16S rRNA gene sequence analysis. Liquid chromatography−mass spectrometry and nuclear magnetic resonance analysis indicated the major pathway of thiacloprid (THI) metabolism by *V. boronicumulans* J1 involved hydrolysis of the *N*-cyanoimino group to form an *N*-carbamoylinino group containing metabolite, THI amide. Resting cells of *V. boronicumulans* J1 degraded 62.5% of the thiacloprid at a concentration of 200 mg/L in 60 h, and 98% of the reduced thiacloprid was converted to the final metabolite thiacloprid amide. A 2.6 kb gene cluster from *V. boronicumulans* J1 that includes the full length of the nitrile hydratase gene was cloned and investigated by degenerate primer polymerase chain reaction (PCR) and inverse PCR. The nitrile hydratase gene has a length of 1304 bp and codes a cobalt-type nitrile hydratase with an α-subunit of 213 amino acids and a β-subunit of 221 amino acids. The nitrile hydratase gene was recombined into plasmid pET28a and overexpressed in *Escherichia coli* BL21 (DE3). The resting cells of recombinant *E. coli* BL21 (DE3)-pET28a-NHase with overexpression of nitrile hydratase transformed thiacloprid to its amide metabolite, whereas resting cells of the control *E. coli* BL21 (DE3)-pET28a did not. Therefore, the major hydration pathway of thiacloprid is mediated by nitrile hydratase.

KEYWORDS: *nitrile hydratase, thiacloprid, thiacloprid amide, Variovorax boronicumulans*

INTRODUCTION

Neonicotinoids are a class of insecticides that act on the central nervous system of insects and may be used for the control of sucking pests such as aphids, whiteflies, leaf- and planthoppers, and thrips. These compounds are the most important new class of synthetic insecticides produced during the past two decades.[1−3] Thiacloprid (THI, Figure 1) was developed in 2000 to control a variety of sucking and chewing insects, primarily aphids and whiteflies. THI is safe for bees, egg parasitoids, and important beneficial organisms; therefore, it can also be sprayed on flowering crops.[4−6]

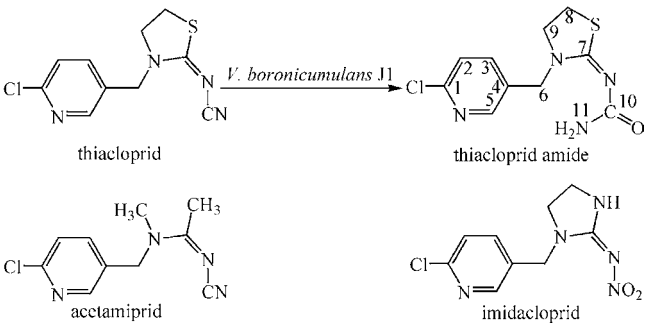

Figure 1. Molecular structures of thiacloprid, acetamiprid, imidacloprid, and its metabolites.

The unique physicochemical properties of neonicotinoid insecticides render them versatile, with application forms such as foliar spray, soil drench, and seed treatment. Among the seven major commercial neonicotinoid insecticides (acetamiprid (ACE), clothianidin, dinotefuran, imidacloprid (IMI), nitenpyram, THI, and thiamethoxam), THI is the only one classified as non-relevant for soil application.[4] Environmental studies explained that THI disappears very rapidly from soils with a half-life of <2 days and that, notably, about 70% of THI was converted to an amide metabolite (THI amide, Figure 1), so that THI is not recommended for soil application.[7−9] We recently proved that soil microbial activity plays a key role in transforming THI to its amide metabolite, and the yeast *Rhodotorula mucilaginosa* strain IM-2 was proved to be able to degrade THI in sucrose mineral salt medium, with 68.6% of reduced THI converted to the amide metabolite.[10] Furthermore, THI amide has a >10-fold lower insecticidal activity but longer persistence in the soil compared to its parent THI, implying that the conversion of THI to THI amide reduces bioefficacy and may produce new environmental risks.[9] Therefore, elucidation of the mechanism of efficient microbial conversion of THI to its amide metabolite may be useful for working out the bioremediation of THI contamination and a suitable technical profile for THI as well as mitigating the possible environmental risk of THI application. As the biotransformation of THI by *R. mucilaginosa* strain IM-2

Received: August 13, 2011
Revised: December 1, 2011
Accepted: December 12, 2011
Published: December 12, 2011

was limited by a slow THI degradation rate with a half-life of 14.8 days and the yeast almost lost its THI hydration activity in a mineral medium without supplementation of sucrose,[9,10] we continued to isolate and screen microbes that could rapidly degrade THI in the soil. In the present study, we report a bacterium strain J1 that transforms THI to its metabolite THI amide with a degradation half-life of 1.8 days and that does not require sucrose for cometabolism of THI.

In previous studies of neonicotinoid insecticides, the enzymatic mechanism of the degradation of IMI has been thoroughly elucidated, but there is a lack of understanding of the mechanisms underlying THI metabolism. Cytochrome P450 enzymes are involved in hydroxylation of IMI and THI at the carbon atom that neighbors the tertiary amine attached to the 6-chloro-3-pyridinylmethyl moiety.[1,11] Aldehyde oxidase, which converts the *N*-nitroimino group of IMI to *N*-nitrosoimino and *N*-aminoimino metabolites, is inactive with respect to the *N*-cyanoimino group of THI.[1] Although the metabolism of THI has been studied in mice, spinach, and soils,[8,12,13] the mechanism of *N*-cyanoimino group metabolism is still poorly understood. In the present study, a nitrile hydratase (EC 4.2.1.84, NHase) coding gene from the isolate J1 was cloned and expressed in *Escherichia coli* BL21 (DE3). Whole cells of *E. coli* BL21 overexpressed NHase could transform THI to the main amide metabolite. Therefore, NHase mediates the degradation of THI to THI amide. To our knowledge, this is the first report of an enzymatic mechanism responsible for microbial degradation of neonicotinoid insecticides. Furthermore, the ability of NHase to transform a nitrile compound to an amide metabolite is first experimentally reported in the genus *Variovorax*.

MATERIALS AND METHODS

Chemicals. THI, IMI, and ACE were provided by the Jiangsu Pesticide Research Institute, Nanjing, China (>97% purity). Reagents for high-performance liquid chromatography (HPLC) analysis were of HPLC grade (E. Merck, Darmstadt, Germany), whereas all other solvents and reagents were of analytical grade from commercial sources.

Medium and Cultivation. The medium used for the isolation of bacteria was Luria−Bertani (LB) containing yeast extract 5 g/L, peptone 10 g/L, and NaCl 10 g/L (pH 7.2). Cultivations were conducted at 30 °C in a 100 mL flask containing 10 mL of LB broth with shaking at 220 rpm. After cultivation for 24 h, 0.1 mL of culture broth was inoculated into a 100 mL flask containing 10 mL of LB broth and incubated under the same conditions.

Identification of the THI-Degrading Microbes. The bacterial isolate was identified by 16S rRNA gene sequence analysis. Nucleotide sequence data were deposited in the GenBank database. In addition, all sequences obtained were compared to sequences already present in the GenBank database using the BLASTN alignment tool. A neighbor-joining phylogenetic tree, based on the sequence of the 16S rRNA gene, was generated by applying MEGA 5.0 software (www.megasoftware.net).

Biotransformation of THI by Resting Cells and Preparation of Metabolites. To evaluate the ability of resting cells to biotransform THI, bacteria were precultivated for 24 h and then harvested by centrifugation at 6000*g* for 5 min. Next, the cell sediments obtained were washed with 0.2 mol/L Na_2HPO_4/NaH_2PO_4 buffer (pH 7.0) and then resuspended in 10 mL of the same buffer. These cell suspensions were used for resting cell transformations. Except where otherwise stated, the initial concentration of THI was 200 mg/L. The transformations were conducted under standard cultivation conditions for the indicated times. Prior to sampling, redistilled water was added to the biodegradation broth up to the initial weight, after which the samples were centrifuged at 10000*g* for 10 min to remove the residual cells and the supernatant was collected, filtered, and diluted to an appropriate volume for analysis of the substrates and metabolites by HPLC.

To prepare the metabolites, biotransformation by the J1 strain was conducted in 1 L flasks containing 300 mL of transformation broth. Following transformation for 60 h, cell residues were removed from the mixture and extracted twice with equal volumes of ethyl acetate. The ethyl acetate fraction was then dehydrated with anhydrous sodium sulfate, after which the organic phase was filtered using an organic membrane with a pore size of 0.22 μm and then concentrated. The product crystals were then repeatedly washed with acetonitrile to eliminate the remaining THI until a purity of >98% was observed upon HPLC analysis, after which the purified metabolite was dried under vacuum.

Cloning the NHase Gene from the J1 Strain. The total genomic DNA from the J1 strain was extracted using a MiniBEST bacterial genomic DNA extraction Kit (TaKaRa, Dalian, China). The degenerate primers P3-f (5′-CCGCCCRCSTGGTACAAGAGC-3′) and P3-r (5′-CGCTCCGGSAASACSAKGTAGC-3′), designed by alignment of the nucleotides of NHase genes from the genus *Variovorax* in the Genbank database, as well as NHCo-f (5′-GTSGTGGCSARGGCCTGG-3′) and NHCo-r (5′-GRKYGCCGATCATCGAGTC-3′), referenced in the study of Precigou et al.,[14] were used for cloning the NHase α-subunit gene. The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. The polymerase chain reaction (PCR) conditions were as reported by Precigou et al.[14] The PCR product was cloned into the pMD18-T vector (TaKaRa, code D101A) and sequenced. Then the new primer NHCo-f (5′-GTCGTGGCGAGGGCCTGG-3′), designed from the determined DNA fragment amplified by using NHCo primers, and the degenerate primer NH-β-r (5′-CTTGCCSYGCACRTAGCC-3′) of the β-subunit gene, referenced in the paper of Precigou et al.,[14] were used for cloning the NHase α- and β-subunit genes. The obtained DNA fragment was further used for cloning the full-length NHase gene. First, inverse PCR was used to clone the 5′-upstream segment of the α-subunit gene. Inverse PCR was performed as described by Ochman et al.[15] Genomic DNA of strain J1 was cut with the appropriate *Pst*I restriction enzyme, incubated at 37 °C for 24 h, and then circularized by ligation with T4 DNA ligase (TaKaRa) at 16 °C for 24 h. The primers NH-αU-f (5′-CGGTGTAGCCCAGCGAGGCGAT-3′) and NH-αU-r (5′-GACCTACACGACGCATGCCGACCT-3′) were used for inverse PCR. The PCR product was ligated by TA cloning and sequenced.

For amplification of the 3′-downstream segment of the NHase β-subunit gene, primers NH-βD-f (5′-CCGAGAACGTGCCCGCTGTGCT-3′), designed from the sequenced DNA, and NH-βD-r (5′-CCGAGAACATCGACACCAAGGCCT-3′), referenced from the determined genomic DNA of *V. paradoxus* S110, were used for PCR, and the PCR product was sequenced.

The nucleotide sequence analysis and multiple sequence alignments were performed by the software DNAMAN version 5.0 (Lynnon Biosoft, Quebec, Canada) and BLAST program in the GenBank database.

Construction of the Expression Vector. To construct the expression vector, primers K9-f (5′-GGGGAATTCATGACCGGCCATGA CCACT-3′) and K9-r (5′-GGGCTCGAGTGCCGCGGGCTCCAGGTA-3′), with the *Eco*RI restriction enzyme site and the *Xho*I restriction enzyme site, respectively, indicated by underscoring, were used for amplification of the full NHase gene involving α- and β-subunit genes, and an Angel *pfu* DNA polymerase (Genscript, Nanjing, China) was used for PCR amplification. Appropriately amplified DNA fragments, excised by restriction digestion with *Eco*RI/*Xho*I restriction enzymes, were cloned between the same restriction sites of the appropriate expression vector pET28a. Ligations were performed according to the manufacturer's (TaKaRa) protocols.

Expression of Recombinant NHase into *E. coli* BL21 (DE3). Preparation of *E. coli* BL21 (DE3) competent cells and electroporation were performed according to the manufacturer's (TaKaRa) protocols. Transformed *E. coli* BL21 (DE3) strains were grown in LB medium supplemented with kanamycin (30 μg/mL) and 0.1 mmol/L $CoCl_2$. Cells were incubated initially at 37 °C on a rotary shaker until the OD_{600} value reached 0.6 and subsequently induced by the addition of 0.1 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG). Afterward,

the cultures were incubated for another 20 h at 20 °C. Crude cell extracts were prepared by sonication and centrifugation at 13000 rpm for 20 min at 4 °C.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE). Samples from cultivation after sonication were divided into soluble protein and total protein fractions and analyzed by SDS-PAGE according to the method of Rzeznicka et al.[16] The standard molecular weight protein mixture was used as reference. Gels were stained for protein detection with Coomassie Brilliant Blue.

HPLC and Liquid Chromatography–Mass Spectrometry (LC-MS) and Nuclear Magnetic Resonance (NMR) Analyses. An Agilent 1200 HPLC system equipped with an HC-C18 column (4.6 × 250 mm, 5 μm particle size, Agilent Technologies, Santa Clara, CA) was employed for analysis of THI and its metabolite. Elution was conducted at a flow rate of 1 mL/min in a mobile phase that contained water and acetonitrile as well as 0.01% acetic acid (water/acetonitrile, 65:35). The signal was monitored at a wavelength of 242 nm using an Agilent G1314A UV detector. LC-MS was conducted using an Agilent LC-MS system equipped with an electrospray ion source that was operated in the positive ion mode. ^{13}C and ^{1}H NMR spectra of the biotransformation product were obtained in DMSO-d_6 using a Bruker AV-400 spectrometer (Switzerland) operating at 100 and 400 MHz, respectively. Chemical shifts were referenced against internal tetramethylsilane.

RESULTS

Isolation and Identification of THI-Degrading Microorganisms from Soils. Soil samples with rapid THI-degrading ability were used to isolate THI-degrading microbes. Twelve bacteria with unique morphologies were observed on the LB plates spread with the above soil suspensions. The abilities of resting cells of these bacteria to transform THI were then evaluated. Among the microbes tested, three strains had the ability to degrade THI, and isolate J1, which had the highest activity, was selected for further study. The J1 strain is Gram-negative and rod-shaped; it has no flagella or spores; its dimensions are 0.5–0.7 × 1.3–1.5 μm. Analysis of the 16S rRNA gene sequence of strain J1 revealed that it was 100% homologous with that of *Variovorax boronicumulans* (accession no. AB300597) (Figure 2). Therefore, isolate J1 was identified as a strain of *V. boronicumulans*, and its sequence was deposited in the GenBank database under accession no. GQ249078. *V. boronicumulans* J1 was also deposited in the China General Microbiological Culture Collection Center (Beijing, China) under the accession no. CGMCC 4969.

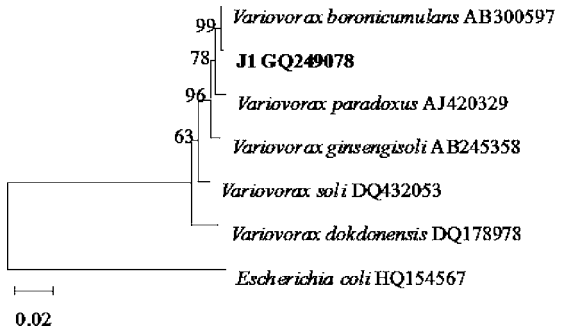

Figure 2. Neighbor-joining phylogenetic tree of strain J1, other members of the genus *Variovorax*, and representatives of some other taxa based on 16S rRNA gene sequence comparisons. Bootstrap percentages from 1000 replicates are shown at nodes. The sequence of *Escherichia coli* was used as the outgroup. Bar = 2% sequence divergence.

Biotransformation of THI by Resting Cells of *V. boronicumulans* J1 and Identification of Metabolites. HPLC analysis (Figure 3) revealed that biotransformation of THI by resting cells of *V. boronicumulans* J1 resulted in the formation of a polar metabolite. The metabolite had a retention time of 4.84 min, but no comparable peak was observed upon analysis of the controls containing the inoculated bacterium alone or the substrate alone. LC-MS analysis indicated that the metabolite displayed a protonated ion (M + H) at m/z 271, a fragment ion (M − NH_2) at m/z 254, and a fragment ion (M − NH_2CO + H) at m/z 228. When compared to the substrate THI (M + H) at m/z 253, the mass of the metabolite was enhanced by the addition of a water molecule. ^{1}H NMR spectral data (DMSO, 400 MHz) of the purified metabolite gave δ 8.41 (s, 1H, C5), 7.82 (d, J = 8.1 Hz, 1H, C3), 7.51 (d, J = 8.1, 1H, C2), 6.53 (s, 1H, N11), 6.28 (S, 1H, N11), 4.71 (S, 2H, C6), 3.53 (t, J = 7.4 Hz, J = 14.8 Hz, 2H, C9), and 3.04 (t, J = 7.4 Hz, J = 14.8 Hz, 2H, C8). ^{13}C NMR spectral data (DMSO, 100 MHz) of the purified metabolite gave δ 168.4 (C7), 163.7 (C10), 150.1 (C5), 149.8 (C1), 140.2 (C3), 132.3 (C4), 124.7 (C2), 49.1 (C6), 46.8 (C9), and 26.6 (C8). The above data were identical with the THI amide reported in our previous study.[10] Therefore, *V. boronicumulans* J1 is able to hydrate THI to form THI amide.

Time course analysis of the hydration of THI by the resting cells of *V. boronicumulans* J1 (Figure 4) indicated that the quantity of THI declined from 0.72 to 0.27 mmol/L in 60 h. The degradation ratio and half-life were calculated as 62.5% and 1.8 days, respectively. In addition, the quantity of THI amide had increased by 0.44 mmol/L, which was equivalent to a mole transformation ratio of 97.8%.

In a previous study,[11] we reported that the biotransformation of THI with hydroxylation by the bacterium *Stenotrophomonas maltophilia* strain CGMCC 1.1788 required carbohydrates as cosubstrates for its degradation. The hydroxylation rate of THI by the resting cells of *S. maltophilia* CGMCC 1.1788 in phosphate buffer was low, whereas it increased 10-fold upon addition of 2% sucrose in phosphate buffer.[11,17] The yeast *R. mucilaginosa* strain IM-2 also has the ability to enhance THI hydration activity in a mineral medium upon the addition of sucrose.[10] However, in this study, the addition of sucrose to the phosphate buffer did not enhance the THI degradation rate or the THI amide formation rate (Table 1); therefore, the degradation of THI and the formation of THI amide by the resting cells of *V. boronicumulans* J1 did not proceed via a cometabolism mechanism. Accordingly, phosphate buffer without additional sucrose was used for subsequent experiments.

In seven commercial neonicotinoid insecticides, ACE has the same pharmacophore *N*-cyanoimino (═N—CN) and 6-chloro-3-pyridinylmethyl moiety as THI (see Figure 1). ACE also rapidly dissipates in soil with a half-life of <8 days.[9,18] *V. boronicumulans* J1 did not degrade or hydrate ACE. ACE and THI differ in that ACE is acyclic and THI has a cyclic thiazolidine. Therefore, the acyclic structure of the ACE molecule may affect the hydrolysis of the *N*-cyanoimino group by *V. boronicumulans* J1. IMI has a molecular structure similar to that of THI, but different pharmacophore groups, with *N*-nitroimino (═N—NO_2) being present in IMI instead of the *N*-cyanoimino (═N—CN) that is present in THI. *V. boronicumulans* J1 did not degrade IMI, which suggests that the *N*-nitroimino group in IMI cannot be attacked by *V. boronicumulans* J1.

Cloning the NHase Gene from *V. boronicumulans* J1 and Bioinformatics Analysis. The strategy for cloning the

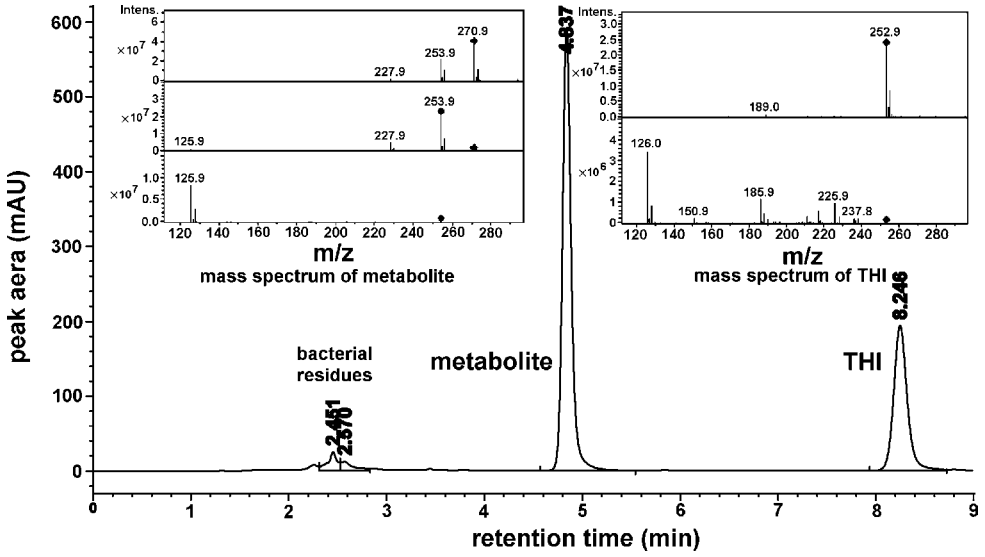

Figure 3. HPLC and LC-MS spectrum of THI metabolite transformed by resting cells of *V. boronicumulans* J1. The sets of substrate THI alone and bacterium alone do not appear at the peak retention time of 4.84 min; therefore, the possibility of spontaneous reaction is excluded.

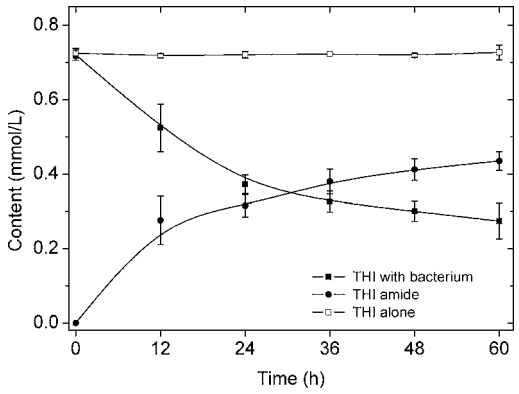

Figure 4. Time course of hydration of THI by resting cells of *V. boronicumulans* J1. The transformation broth without inoculated bacterium does not degrade THI; therefore, the hydration of THI primarily occurs in response to metabolism by *V. boronicumulans* J1. The optical density value at 600 nm for the transformation of resting cells was 5. Bars indicate the standard deviation (SD), $n = 9$.

NHase coding gene from *V. boronicumulans* J1 is shown in Figure 5. First, the 224 bp length of the NHase gene (accession no. AJ577856) from *Variovorax* sp. DSM 11402 and the NHase genes Vapar_2111 and Vapar_1665 from the whole genome determined as *V. paradoxus* S110 (accession no. NC_012791) were aligned using the ClustalX multiple-sequence alignment program. The degenerate primers P3-f and P3-r designed from the alignment result were used for cloning the NHase α-subunit gene, and a 156 bp DNA fragment was amplified and sequenced.

The nucleotide BLAST program indicated that the 156 bp DNA fragment from *V. boronicumulans* J1 is a partial putative NHase α-subunit gene that has 93% identity with the NHase gene from *Variovorax* sp. DSM 11402. The above results indicated that there is a NHase coding gene in the genomic DNA from *V. boronicumulans* J1. The pair of P3 primers was used to check the PCR specificity of the NHase α-subunit gene in the subsequent PCR amplification and TA cloning. Meanwhile, a partial NHase α-subunit gene with a length of 421 bp was obtained from the PCR amplification by using the degenerate primers NHCo-f and NHCo-r. Subsequently, an extended 927 bp DNA fragment involving partial α- and β-subunit coding genes was amplified by using primers NHCo-f and NH-β-r. A product containing 1009 bp of DNA was obtained by inverse PCR using primers NH-αU-f and NH-αU-r. A 989 bp DNA fragment was amplified using primers NH-βD-f and NH-βD-r.

DNA fragments of lengths 927, 1009, and 989 bp were assembled to form a DNA fragment of 2629 bp. This 2629 bp DNA consists of a partial thymidylate synthase gene, a NHase gene, and a hypothetical protein coding gene (Figure 5). The NHase gene comprises 1304 bp and is composed of an α-subunit of 642 bp (739−1380) and a β-subunit of 666 bp (1377−2042, Figure 5). The accession numbers of the NHase α- and β-subunit coding genes from *V. boronicumulans* J1 in the Genbank database are JN243858 and JN243859, respectively.

The putative translation product of the α-subunit gene consists of 213 amino acids, and its highest identity (87%) is to the NHase α-subunit of *V. paradoxus* EPS (YP_004154136);

Table 1. Effect of Sucrose on Biotransformation of THI by Resting Cells of *V. boronicumulans* J1[A]

	concentration (mmol/L)			
additional sucrose	reduced THI	formed THI amide	THI degradation rate (%)	molar transformation rate (%)
−	0.45 ± 0.05*	0.44 ± 0.03*	62.5	97.8
+	0.41 ± 0.03*	0.40 ± 0.03*	59.4	97.6

[A]To test the effect of sucrose on the biotransformation of THI, 2% sucrose (w/v) was added to the resting cell transformation buffer. The optical density at 600 nm was 5. The reaction time was 60 h. The THI degradation rate was calculated from the ratio of the reduced amount to the initial amount of THI, and the THI amide formation rate was calculated from the ratio of the formed THI amide amount to the reduced THI amount. Data are expressed as the mean ± standard deviation (SD), $n = 9$. *, $p > 0.05$.

dx.doi.org/10.1021/jf203232u | J. Agric.Food Chem. 2012, 60, 153−159

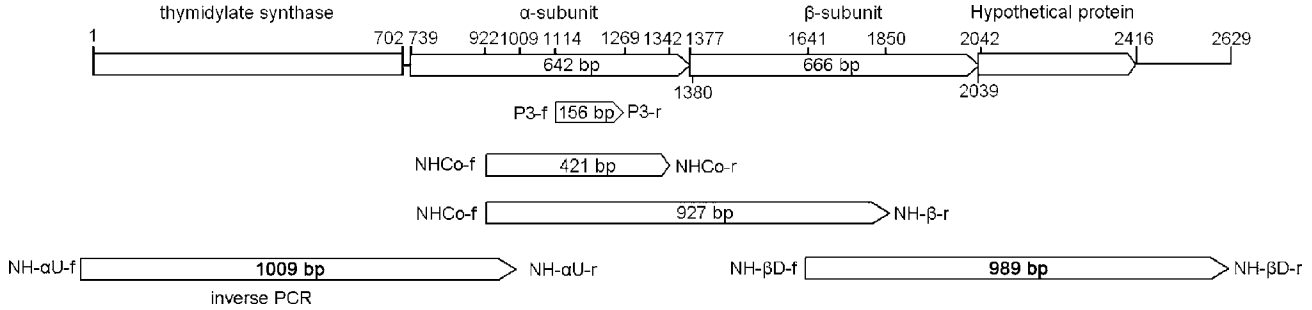

Figure 5. Strategy for cloning the NHase coding gene from *V. boronicumulans* J1. The location of primers in the NHase coding gene is marked in the top line.

the putative translation product of the β-subunit gene consists of 221 amino acidsm and its highest identity (87%) is to the NHase β-subunit of *V. paradoxus* S110 (YP_002943582). A highly conserved motif in the α-subunit is composed of the amino acid sequence Val-Cys-Thr-Leu-Cys-Ser-Cys. The fact that the third amino acid is threonine, not serine, signifies that the NHase from *V. boronicumulans* J1 is a cobalt-type NHase, not an iron-type NHase.[19,20] This is consistent with the experimental result of >2-fold stimulation by cobalt of the THI degradation rate and THI amide formation achieved by *V. boronicumulans* J1 compared to the control with no cobalt stimulation (Table 2).

Table 2. Resting Cell Transformation of THI by the Wild *V. boronicumulans* J1 and the Recombinant *E. coli* BL21 (DE3) pET28a-NHase and the Effect of Cobalt on Biotransformation of THI by *V. boronicumulans* J1[a]

	content (μmol/L)		
cell	initial THI	THI at 24 h	THI amide at 24 h
V. boronicumulans J1	83.5 ± 6.9	42.6 ± 0.7	39.1 ± 0.1
V. boronicumulans J1 without cobalt stimulation	79.9 ± 4.4	60.7 ± 4.8	15.3 ± 1.1
pET28a-NHase	84.4 ± 1.7	50.0 ± 3.2	31.2 ± 0.3
pET28a	79.9 ± 7.8	79.3 ± 6.5	ND

[a]The initial concentration of THI was set as 20 mg/L. The NHase coding gene was inserted into vector pET28a, and the recombinant vector was transformed into *E. coli* BL21 (DE3). NHase was expressed by induction with 0.1 mmol/L IPTG, and $CoCl_2$ was added into the LB cultivation broth for stimulation of NHase. The cell optical density at 600 nm was 10. Data are expressed as the mean ± SD, n = 9. ND, no detection.

Expression and Functionality of the NHase from *V. boronicumulans* J1 in *E. coli* BL21 (DE3). The recombinant vector pET28a-NHase was transformed into *E. coli* BL21 (DE3), and the cells were induced by 0.1 mmol/L IPTG. SDS-PAGE analysis (Figure 6) indicated that the NHase was expressed in *E. coli* BL21 (DE3) pET28a-NHase (lane 3), whereas it was not in control experiments with *E. coli* BL21 (DE3) and *E. coli* BL21 (DE3) pET28a (lanes 1 and 2, respectively). The expressed α- and β-subunits had similar predicted molecular weights of 27 kDa, and therefore a single mixed band of total proteins (lane 3) and soluble proteins (lane 7) was observed in SDS-PAGE.

Transformation of THI by the resting cells of *E. coli* BL21 (DE3) pET28a-NHase with overexpression of NHase indicated that THI was converted to THI amide according to HPLC, LC-MS, and NMR analyses (Figure 7), whereas cells without the

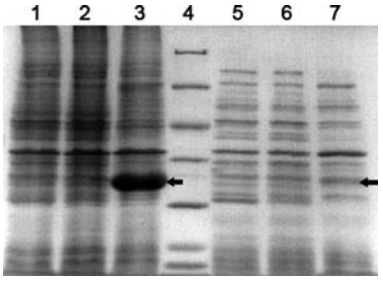

Figure 6. SDS-PAGE of the overexpressed NHase gene in *E. coli* BL21 (DE3). Lanes: 1, total proteins of *E. coli* BL21 (DE3); 2, total proteins of *E. coli* BL21 (DE3) pET28a; 3, total proteins of *E. coli* BL21 (DE3) pET28a-NHase; 4, standard protein markers (116.0, 66.2, 45.0, 35.0, 25.0, 18.4, and 14.4 kDa); 5, soluble proteins of *E. coli* BL21 (DE3); 6, soluble proteins of *E. coli* BL21 (DE3) pET28a; 7, soluble proteins of *E. coli* BL21 (DE3) pET28a-NHase.

recombinant NHase gene did not transform THI. After transformation for 24 h, the cells of *E. coli* BL21 (DE3) pET28a-NHase degraded 40.7% of THI and formed 31.2 μmol/L of THI amide, whereas the control *E. coli* BL21 (DE3) pET28a cells did not degrade THI or form THI amide (Table 2). Therefore, the major hydration pathway in the biotransformation of THI by *V. boronicumulans* J1 is mediated by a NHase. Under the same conditions, wild *V. boronicumulans* J1 degraded 48.9% of THI and formed 39.1 μmol/L of THI amide. Obviously, the recombinant NHase showed a slightly lower activity than the wild strain. Interestingly, the expressed NHase exhibited no THI hydration activity without $CoCl_2$ addition to the culture LB broth, which indicates that cobalt is necessary for NHase activity. In results identical to the experiments testing biotransformation of ACE and IMI by the resting cells of *V. boronicumulans* J1, the recombinant *E. coli* BL21 (DE3) pET28a-NHase with overexpressed NHase did not transform ACE or IMI.

DISCUSSION

In neonicotinoid chemistry, the selectivity of neonicotinoids to insect and vertebrate nicotinic acetylcholine receptor (nAChR) is mainly attributed to the pharmacophore *N*-nitroimino or *N*-cyanoimino group, and slight alterations to the pharmacophore can completely reverse the selective toxicity of neonicotinoids for insects over vertebrates.[21] Therefore, biotransformation of the neonicotinoid pharmacophore moiety has been a particular focus in plant and mammalian systems.[1] However, there have to date been no reports on the enzymatic metabolism of the *N*-cyanoimino group in neonicotinoid insecticides. Our present work indicates that a NHase mediates the hydrolysis of THI to

dx.doi.org/10.1021/jf203232u | J. Agric.Food Chem. 2012, 60, 153–159

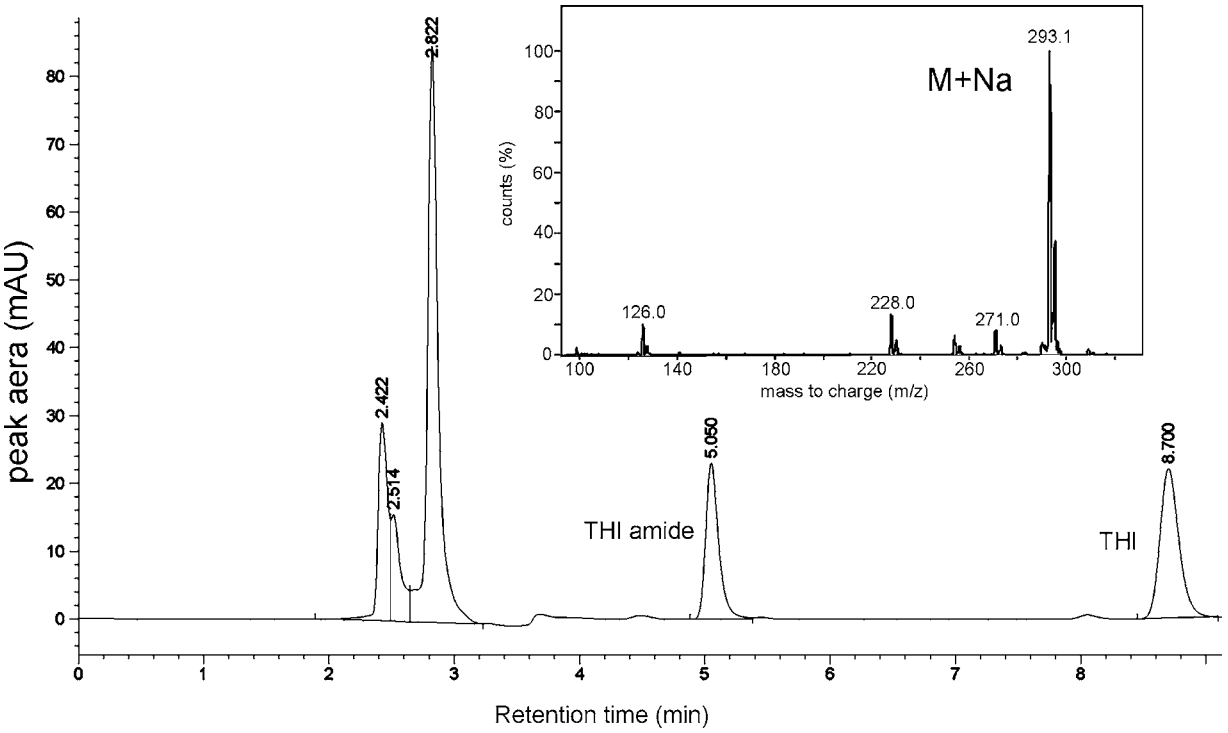

Figure 7. HPLC and LC-MS spectrum of biotransformation of THI by the resting cells of *E. coli* BL21 (DE3) overexpressed the NHase from *V. boronicumulans* J1. The concentration of THI was set as 20 mg/L, the reaction time was 24 h, and the cell density was 10. The resting cells of *E. coli* BL21 without recombinant NHase coding gene did not transform THI. A C18 precolumn was fixed before the HPLC column; therefore, delayed retention time of THI amide and THI was observed.

THI amide, rather than the aldehyde oxidase that affects the biotransformation of IMI at the *N*-nitroimino group. In addition, NHase-encoding genes are abundant in soil samples;[14] therefore, THI is easily and rapidly converted to the amide metabolite, which has 10-fold lower insecticidal activity, by NHases from soil samples and soil microbes when THI is used for soil application. Interestingly, ACE has the same *N*-cyanoimino group as THI and also dissipates rapidly in soil.[18,22] *V. boronicumulans* J1 and the *E. coli* BL21 (DE3)-expressed NHase were not capable of degrading ACE. The proposed metabolic pathway of ACE is via oxidative cleavage of the *N*-cyanoimino group to form the urea metabolite IM 1-3 in the honeybee[23] or via cleavage at the $N(CH_3)-C(CH_3)CN$ linkage to form metabolite IM 1-4, which is subsequently acetylated to IM 1-3 in mice.[12] Apparently, the two metabolic pathways involve cleavage of ACE, not hydration of the *N*-cyanoimino group to an amide metabolite by a NHase.

Co-metabolism is frequently observed during the transformation of xenobiotic nongrowth substrates by microbes.[24,25] Recently, a few pure microbe cultures, such as *Pseudomonas* sp. G1 and *Leifsonia* sp. PC-21, were proved to be capable of degrading neonicotinoid insecticides. The two bacteria cometabolically transform IMI with supplementation of glucose in the culture medium.[26,27] We previously reported that *S. maltophilia* CGMCC 1.1788 degraded THI via hydroxylation at the 4-carbon atom of the thiazolidine ring.[11] Furthermore, the addition of sucrose to the phosphate buffer enhanced the hydroxylation of THI. During hydroxylation, sucrose does not function as a carbon source for cell growth but, rather, as an energy source for the regeneration of cofactor NAD(P)H.[11,28] The yeast *R. mucilaginosa* IM-2 degraded THI when grown in a special sucrose mineral salt medium, whereas it degraded much less THI in the absence of additional sucrose. The mechanism by which sucrose enhances THI degradation by *R. mucilaginosa* IM-2 is still unclear.[10] In the present study, NHase is proved to mediate the hydrolysis of THI to form the amide metabolite, and the reaction catalyzed by NHase does not require a cofactor such as NAD(P)H for hydration.[29] Therefore, *V. boronicumulans* J1 achieved greater and more rapid hydrolysis of THI in a phosphate buffer without sucrose.

NHases catalyze the conversion of nitrile compounds to the corresponding amides and are important for industrial purposes. NHases have been discovered in several genera of microbes, such as *Agrobacterium*, *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Nocardia*, *Pseudomonas*, *Pseudonocardia*, *Rhodococcus*, *Candida*, and *Cryptococcus*.[30] In the genus *Variovorax*, the NHases were only bioinformatically predicted from the determined genomic DNA of *V. paradoxus* EPS and *V. paradoxus* S110 as well as from the partial NHase gene of 224 bp length from *Variovorax* sp. DSM 11402 that was cloned by Lourenco et al.[31] Nielson et al.[32] reported that *Variovorax* sp. DSM 11402 transformed the herbicides ioxynil and bromoxynil, which contain nitrile moieties, into their corresponding amides and carboxyl metabolites. However, the NHase gene from *Variovorax* sp. DSM 11402 has not been cloned nor has the enzymatic function of the NHase been examined. Our present work is the first to experimentally prove that the NHase from *V. boronicumulans* J1 can transform the neonicotinoid insecticide THI to THI amide. The metabolism of nitrile compounds usually involves hydration by NHase to produce an amide that is subsequently converted to a carboxyl metabolite via an amidase.[33] *V. boronicumulans* J1 was capable of hydrolyzing THI to THI amide at a conversion ratio of 98%, but the carboxyl metabolite was not detected upon HPLC or LC-MS analysis, implying that *V. boronicumulans* J1 cannot further transform THI amide to the carboxyl metabolite.

Because the full length of the NHase gene from the genus *Variovorax* has for the first time been cloned and expressed, its potential applications for industrial biotransformation and environmental bioremediation of nitrile compounds will be assessed in further study.

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Funding

This research was financed by the Priority Academic Program Development of Jiangsu Higher Education Institutions, the National Science Foundation of China (30970040), and the Program of Jiangsu Higher Education Institution of China (09KJB180003).

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