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ORIGINAL PAPER

Degradation of methamidophos by *Hyphomicrobium* species MAP-1 and the biochemical degradation pathway

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Abstract Methamidophos is one of the most widely used organophosphorus insecticides usually detectable in the environment. A facultative methylotroph, *Hyphomicrobium* sp. MAP-1, capable of high efficiently degrading methamidophos, was isolated from methamidophos-contaminated soil in China. It was found that the addition of methanol significantly promoted the growth of strain MAP-1 and enhanced its degradation of methamidophos. Further, this strain could utilize methamidophos as its sole carbon, nitrogen and phosphorus source for growth and could completely degrade 3,000 mg l^{-1} methamidophos in 84 h under optimal conditions (pH 7.0, 30°C). The enzyme responsible for methamidophos degradation was mainly located on the cell inner membrane (90.4%). During methamidophos degradation, three metabolites were detected and identified based on tandem mass spectrometry (MS/MS) and gas chromatography-mass spectrometry (GC–MS) analysis. Using this information, a biochemical degradation pathway of methamidophos by *Hyphomicrobium* sp. MAP-1 was proposed for the first time. Methamidophos is first cleaved at the P–N bond to form *O,S*-dimethyl hydrogen thiophosphate and NH_3. Subsequently, *O,S*-dimethyl hydrogen thiophosphate is hydrolyzed at the P–O bond to release $–OCH_3$ and form *S*-methyl dihydrogen thiophosphate. *O,S*-dimethyl hydrogen thiophosphate can also be hydrolyzed at the P–S bond to release $–SCH_3$ and form methyl dihydrogen phosphate. Finally, *S*-methyl dihydrogen thiophosphate and methyl dihydrogen phosphate are likely transformed into phosphoric acid.

Keywords Biodegradation pathway · Facultative methylotroph · *Hyphomicrobium* sp. MAP-1 · Methamidophos

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Introduction

The insecticide methamidophos (*O,S*-dimethyl phosphoramidothioate) is highly effective at killing a variety of pests and has therefore been applied globally in agricultural production (de Castro and Chiorato 2007). The amount of methamidophos applied to agricultural fields reached up to 3.5×10^4 t in 1990 in

China, making it the top ranking insecticide used (Lin et al. 2000). Methamidophos has a high solubility in water (>2,000 g l^{-1}, 25°C) and is considered one of the most toxic insecticides; it can cause acute toxic effects by the inhibition of acetylcholinesterase (Sultatos 1994) and can significantly damage peoples' health (Temerowski and van der Staay 2005). Recently, methamidophos has been prohibited or its application restricted to only a few crops in many countries, including China. Even though methamidophos has a relatively short half-life in anaerobic (<5 days) and aerobic (>41 days) soils (EPA 1998), the amount of methamidophos residues always exceeds the self-purifying capacity of studied soil ecosystems (Garci'a-de la Parra et al. 2006; Zhou and Wang 2006). As a result, methamidophos was often detected in high concentrations in the ecosystem and thus lead to some direct and potential adverse effects on environmental safety (Battershill et al. 2004; Wang et al. 2008).

The bioremediation of pesticide-contaminated sites is recognized as a cost-effective and reliable method, and studies of the biodegradation of methamidophos have been carried out previously. Several microorganisms such as *Pseudomonas* sp. WS-5 (Xiao and Wang 1995), *Aspergillus orantus* (Liu and Zhong 1999), *Aspergillus orantu* sp. M-2 (Li et al. 1999), *Aeromonas* sp. WB-1 (Chao et al. 1999, 2000), *Saccharomyces rouxii* WY-3 (Liu et al. 2001), *Pseudomonas* sp. S-2 (Wu et al. 2005), *Rhodopesudomonas plaustris* sp. HP-1 (Zhang et al. 2005) and *Acinetobacter* sp. HS-A32 (Zheng et al. 2006) that are capable of degrading methamidophos have been reported. To date however, only a few simple inorganic metabolites such as CH_3OH, PO_4^{3-} and NH_4^+ have been identified during methamidophos transformation (Liu et al. 2001; Malato et al. 1999; Wu et al. 2005; Zheng et al. 2006), and there is still no clear pathway for the biochemical degradation of methamidophos.

In this study, the degrading characteristics of methamidophos by a newly isolated *Hyphomicrobium* species MAP-1 were studied and a biochemical degradation pathway of methamidophos was proposed for the first time. This paper details a potentially exciting bacterium for the bioremediation of methamidophos contamination and proposes a detailed mechanism for methamidophos degradation in microorganisms.

Materials and methods

Chemicals and media

Methamidophos (>99% purity) was purchased from the Pesticide Research Institute (Shanghai, China). A 50,000 mg l^{-1} stock solution of methamidophos was prepared in double-distilled water. High-performance liquid chromatography grade methanol, dichloromethane and acetone were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other reagents used in this study were of analytical reagent grade.

Mineral salts medium (MSM-1 medium: 1.50 g $K_2HPO_4{\cdot}H_2O$, 0.50 g KH_2PO_4, 0.50 g NaCl, 0.20 g $MgSO_4{\cdot}7H_2O$, 0.25 mg $CaC1_2{\cdot}2H_2O$, 3.50 mg $FeC1_2{\cdot}4H_2O$, 0.20 mg $ZnSO_4{\cdot}7H_2O$ per liter) (Nikolausz et al. 2005) supplemented with methamidophos was used to enrich and isolate methamidophos-degrading strains. To study whether the isolated strain could utilize methamidophos as its sole carbon, nitrogen and phosphorus source for growth, MSM-2 medium (0.50 g NaCl, 0.20 g $MgSO_4$, 25 mg $CaC1_2{\cdot}2H_2O$, 3.5 mg $FeC1_2{\cdot}4H_2O$, 0.20 mg $ZnSO_4{\cdot}7H_2O$ per liter) supplemented with 1,000 mg l^{-1} methamidophos was used. For optimal degradation condition experiments, MSM-3 (MSM-1 medium supplemented with 1.0 g l^{-1} NH_4NO_3) and MSM-4 medium (MSM-3 medium supplemented with 0.5% (v/v) methanol) were used.

Strain isolation and characterization

Long-term methamidophos-contaminated soil was collected from the Dongjing Pesticide Factory in Jiangsu province, China, which produced methamidophos for many years. Five grams of soil were added to 50 ml MSM-1 medium containing 1,000 mg l^{-1} methamidophos and incubated in a rotary shaker at 170 rpm at 30°C. Every 7 days, 5 ml of the enriched samples were transferred to 50 ml fresh MSM-1 medium with a stepwise increase in methamidophos concentration to 3,000 mg l^{-1}. Dilutions of the sequential enrichments were plated on MSM-1 agar plates containing 1,000 mg l^{-1} methamidophos, and bacterial colonies were tested for their methamidophos-degrading capabilities. One strain, designated MAP-1, which possessed the highest methamidophos-degrading ability among the isolated strains, was selected for further study.

The isolated strain was identified based on its morphological, physiological and biochemical properties (with reference to *Bergey's Manual of Determinative Bacteriology* (Holt et al. 1994)), combined with 16S rRNA gene sequence analysis. Cell morphology was examined by light microscopy (BH-2; Olympus) and transmission electron microscopy (TEM) (H-7650, Hitach) using cells from an exponentially growing culture. The MAP-1 16S rRNA gene was amplified by PCR with the primer pair 20F (5′-agagtttgatcctggctcag-3′)/1500R (5′-ggttaccttgttacgactt-3′) (Yoon et al. 1996). The 1,365-bp PCR product was sequenced by TaKaRa Biotechnology (Dalian) and deposited in the GenBank database under accession number GQ131420. Sequences were analyzed using the BLASTN search tools (http://www.ncbi.nlm.nih.gov/blast). Alignments with different 16S rRNA gene sequences from GenBank were performed using Clustal X 1.8.3 with default settings. Phylogenesis was analyzed with MEGA version 3.0 software and distances were calculated using the Kimura 2 parameter distance model. A phylogenetic tree was built using the neighbor joining method. Each dataset was bootstrapped 1,000 times.

The effect of different C_1-compounds on the growth of strain MAP-1

MAP-1 cells were pre-cultured in MSM-3 medium containing 1,000 mg l^{-1} methamidophos at 30°C for 48 h and harvested by centrifugation at 5,000 rpm for 5 min. The cell pellet was washed twice with sterile water, and the cell density was adjusted to $OD_{660nm} = 0.60$. One milliliter of the above cells was inoculated into 50 ml sterilized MSM-3 medium containing 1,000 mg l^{-1} methamidophos. Various C_1-compounds were added as the additional carbon sources as follows: 0.1% (v/v) methanol, 0.1% (w/v) sodium formate and 0.1% (w/v) methylamine·HCl. Different concentrations of methanol (0.05, 0.1, 0.5, 1.0 and 2.0% (v/v)) were also used to study the effect of methanol concentration on the growth of MAP-1. Cultures were regularly checked for the MAP-1 growth, and the cell density was monitored by measuring the absorbance at 660 nm using a SHIMADZU UV–Vis recording spectrophotometer. All of the treatments were replicated three times.

Degrading characteristics of MAP-1

To study the methamidophos degrading characteristics of MAP-1, 1 ml of pre-cultured MAP-1 cells ($OD_{660nm} = 0.60$) was inoculated into 50 ml of media (indicated below) containing 1,000 mg l^{-1} methamidophos. The media used were as follows: (1) MSM-2 medium, where methamidophos was the sole carbon, nitrogen and phosphorus source; (2) MSM-3 medium, where 1.0 g l^{-1} NH_4NO_3 was used as an additional nitrogen source; (3) 0.5% (v/v) methanol added to MSM-1 medium as an additional carbon source; (4) 0.5% (v/v) methanol and 1.0 g l^{-1} NH_4NO_3 added to MSM-2 as additional carbon and nitrogen sources; and (5) MSM-4 medium, where 0.5% (v/v) methanol and 1.0 g l^{-1} NH_4NO_3 were used as additional carbon and nitrogen sources. The cultures were incubated with shaking at 170 rpm at 30°C and were regularly monitored for MAP-1 growth and methamidophos degradation. The degradation efficiency was determined by estimating the removal of methamidophos from the culture. As a control, medium inoculated with heat-killed MAP-1 cells was maintained according to the same conditions. All of the treatments were replicated three times.

MSM-4 medium was used to study the effects of environmental factors on the degradation of methamidophos by MAP-1. To investigate the effect of temperature on methamidophos biodegradation at pH 7.0, culture was incubated at 20, 25, 30 and 37°C. To investigate the effect of initial pH value on methamidophos biodegradation at 30°C, the initial pH value of the medium was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0 or 9.0 by changing the potassium-phosphate buffer system. To investigate the effect of initial concentration of methamidophos on its biodegradation at pH 7.0 and 30°C, 1,000, 2,000, 3,000, 4,000 and 5,000 mg l^{-1} methamidophos were added to the MSM-4 medium, respectively.

To determine if MAP-1 could degrade other organophosphorus insecticides such as acephate, chloramidophos, optunal, triazophos, chlorpyrifos, dimethoate, methyl-parathion, fenitrothion, fenamiphos, isofenphos-methyl and dichlorvos, these compounds were added to MSM-4 medium at a concentration of 100 mg l^{-1}. The concentrations of these pesticides remaining after 5 days of MAP-1 growth were determined as previously described (Hong et al. 2007; Hung

et al. 2004). All of the treatments were replicated three times.

Cellular location of the MAP-1 methamidophos-degrading enzyme

To determine the cellular location of the enzyme responsible for methamidophos degradation in MAP-1, cells were grown to late-exponential phase in MSM-4 medium containing 1,000 mg l^{-1} methamidophos. Medium, periplasmic, cytoplasmic, cell inner membrane and outer membrane fractions were prepared as previously described (Ferreira et al. 1990; Myers and Myers 1992). The methamidophos-degrading enzyme activity was assayed in reactions containing 0.1 ml of the crude enzyme solution, 4.8 ml of 50 mM potassium phosphate buffer (pH 7.0) and 1,000 mg l^{-1} methamidophos. The mixture was incubated at 30°C for 15 min. The reaction was stopped by the addition of 0.1 ml of 1 M HCl. The methamidophos-degrading activity was quantified by measuring the amount of methamidophos removed in 1 min. All enzyme assays were run in triplicate.

Analysis of methamidophos

Methamidophos was extracted from the media with three volumes of acetone. The extract was dried over anhydrous Na_2SO_4 and concentrated to 1 ml under reduced pressure using a centrifugal evaporator at room temperature. All samples were analyzed by gas chromatography (GC) (Shimadzu GC-14B) coupled with a nitrogen phosphorus detector (NPD). Nitrogen was used as the carrier gas (36 ml min^{-1}), and the compounds were eluted on an OV-17 capillary column, with the temperatures of the injector, detector and column set at 230, 250 and 200°C, respectively.

Identification of the metabolites during methamidophos biodegradation

To collect metabolites for qualitative analysis, MAP-1 cells were inoculated into 50 ml MSM-2 medium containing 1,000 mg l^{-1} methamidophos. A negative control was similarly prepared except that the inoculated cells were heat-killed. To extract the metabolites, culture samples were collected when the degrading percentages of methamidophos were about 30, 50 and 70%. The three samples were pooled and extracted with an equal volume of dichloromethane. Subsequently, the residual aqueous phase was extracted with an equal volume of acetone. All of the extracts were then evaporated as described previously and re-dissolved in a total of 2 ml of methanol. The combined extracts were then separated and identified by tandem mass spectrometry (MS/MS) (Finnigan, USA) and GC–MS (Finnigan, USA).

In the MS/MS analysis, the metabolites were confirmed by standard MS, ionized by electrospray with a negative polarity, and scanned in the normal mass range from 80 m/z (mass to charge ratio) to 145 m/z. Characteristic fragment ions were detected with second-order MS. The GC–MS analyses were performed in electron ionization (EI) mode (70 eV) with a Finnigan GC, equipped with an MS detector. A Finnigan capillary column (15 m length × 0.5 mm id × 0.25 μm film thickness) was used with a temperature program of: 50°C for 1 min; increased to 140°C at 10°C min^{-1} and held for 1 min; and finally increased to 240°C at 50°C min^{-1} and held at 240°C for 1 min. Helium was used as the carrier gas at a constant flow of 1.0 ml min^{-1}. The samples were analyzed in split mode (1:20) at an injection temperature of 220°C and an EI source temperature of 250°C and scanned in the mass range from 30 to 250 m/z.

Results

Isolation and characterization of the methamidophos-degrading strain MAP-1

Several bacterial strains were isolated from the long-term methamidophos-polluted soil samples by the enrichment culture technique. All isolates were tested for their methamidophos-degrading capacities. One strain designated as MAP-1, which could completely degrade 1,000 mg l^{-1} methamidophos in 84 h in MSM-3 medium, was selected.

MAP-1 did not grow in rich media such as nutrient broth or peptone broth and grew slowly in MSM-3 medium (1,000 mg l^{-1} methamidophos). However, the addition of methanol to MSM-3 medium greatly enhanced its growth. Colonies of MAP-1 on MSM-3 agar are raised, entire, white and 0.5–1 mm in diameter after 7 days of incubation at 30°C. The

morphology of MAP-1 cells is rod-shaped, oval or bean-shaped (0.3–0.6 μm in width and 1.0–2.5 μm in length) with a polar hyphal filament (prostheca). It is non-spore-forming, Gram-negative, polarly flagellated, appendaged and reproduced by budding. MAP-1 showed positive reactions for catalase, oxidase and nitrate reduction and showed negative reactions for indole production, hydrogen sulfide production, gelatin hydrolysis, starch hydrolysis, methyl red test or Voges-Proskauer test. It did not metabolize glucose oxidatively or fermentatively. We found that MAP-1 utilized a limited number of carbon sources: acetate, methanol, sodium formate and methylamine·HCl. It was unable to grow on succinate, D-xylose, D-glucose, D-mannose, galactose, maltose, sucrose, lactose, D-sorbitol, D-mannitol, inositol, glycerol, soluble starch or citrate. MAP-1 could use ammonia and nitrate as the sole sources of nitrogen but could not grow on urea. The optimal temperature and pH value for MAP-1 growth were 25–30°C and pH 6–8, respectively.

We constructed a phylogenetic tree based on the MAP-1 16S rRNA gene sequence (Fig. 1). MAP-1 was closely clustered with *Hyphomicrobium denitrificans* DSM 1869^T, with a sequence similarity score of 99.6%. Based on the above phenotypic characteristics and phylogenetic analysis, strain MAP-1 was identified as *Hyphomicrobium* species.

The effects of different C_1-compounds on MAP-1 growth

It has been reported that supplementing growth media with certain C_1 compounds stimulates the development of *Hyphomicrobia*. To find a suitable medium for MAP-1 growth and to design efficient conditions to accelerate methamidophos degradation, 0.1% (v/v) methanol, 0.1% (w/v) sodium formate and 0.1% (w/v) methylamine·HCl were added to MSM-3 medium (1,000 mg l^{-1} methamidophos), respectively. It was found that all of these C_1 compounds promoted the growth of MAP-1. Compared to sodium formate and methylamine·HCl, 0.1% (v/v) methanol had the greatest stimulatory effect on MAP-1 growth. Subsequently, the effect of methanol concentration on MAP-1 growth showed that 0.5–2% (v/v) methanol could increase the OD_{660nm} of cells from 0.01 to 1.91 in 96 h. Because there were no significant differences in the growth stimulation effect between 0.5, 1 and 2% (v/v) methanol, the optimal methanol concentration for supporting MAP-1 growth was selected as 0.5% (v/v).

MAP-1 methamidophos degrading characteristics

In MSM-2, the cell density of MAP-1 increased from 0.01 to 0.08 (OD_{660nm}) in 96 h accompanied by a

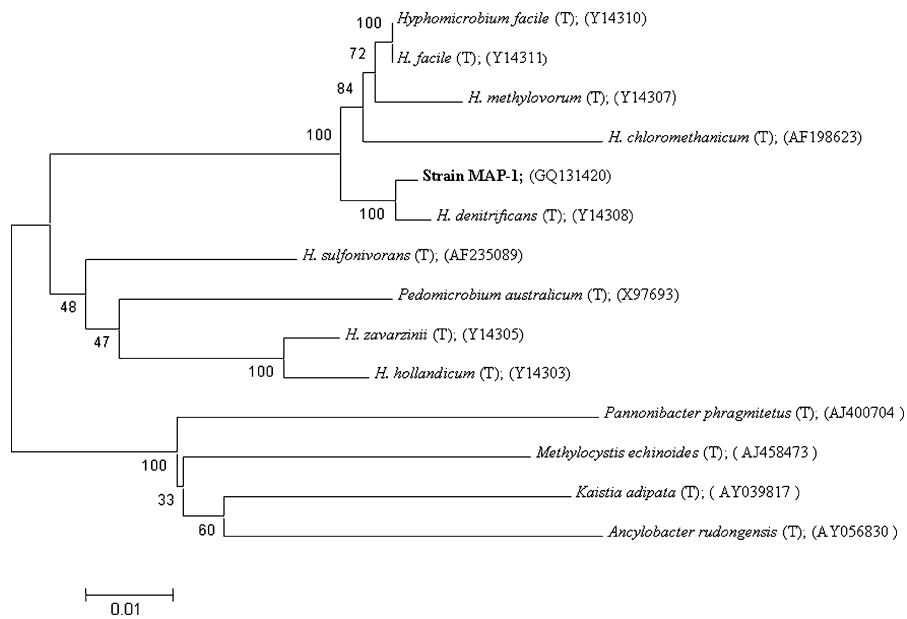

Fig. 1 Phylogenetic analysis of MAP-1 and related species by the neighbor-joining approach. Bootstrap values obtained with 1,000 resamplings are indicated as percentages at all branches. The *scale bars* represent 0.01 substitutions per nucleotide position. The GenBank accession number for each strain is shown in parentheses after the species name

decrease of methamidophos from 1,000 to 890 mg l^{-1}, indicating that MAP-1 could utilize methamidophos as the sole carbon, nitrogen and phosphorus source for growth. In MSM-4, 1,000 mg l^{-1} methamidophos were degraded to an non-detectable level in 36 h, and the cell density increased from 0.01 to 1.73 (OD_{660nm}). In MSM-3 and MSM-1 media, there were delays in the degradation of methamidophos compared to that in MSM-4; MAP-1 required 84 and 72 h, respectively, to completely degrade 1,000 mg l^{-1} methamidophos. In MSM-2 supplemented with 0.5% (v/v) methanol and 1.0 mg l^{-1} NH_4NO_3, MAP-1 required an even longer time to completely remove the methamidophos, indicating that this strain preferred to use methamidophos as a nitrogen source instead of a carbon and/or phosphorus source.

Strain MAP-1 could efficiently degrade methamidophos in the temperature range of 25–30°C and pH value range of 6.0–8.0. The optimum degradation temperature and pH value were 30°C and 7.0, respectively. Under these conditions, MAP-1 was able to completely degrade 1,000, 2,000 and 3,000 mg l^{-1} methamidophos in 36, 60 and 84 h, respectively. Surprisingly, MAP-1 could degrade nearly 75% of methamidophos in 96 h when the concentration was up to 4,000 mg l^{-1}. However, higher concentrations of methamidophos are toxic to the microbes; 5,000 mg l^{-1} methamidophos severely inhibited the biodegradation. These results illuminate that MAP-1 has a great potential in the bioremediation of relatively high concentrations of methamidophos in contaminated sites.

It was interesting to note that MAP-1 can significantly degrade acephate, chloramidophos, optunal, fenamiphos and isofenphos-methyl which all have the P–N bond (Table 1). These results indicate that MAP-1 has a broad substrate range and has the ability to break down the P–N bond of some organophosphorus pesticides.

Table 1 Degradation of some organophosphates pesticides (100 mg l^{-1}) by strain MAP-1 in 5 days

Substrate	OD (660 nm)	Degradation rate (mg l^{-1} d^{-1})
Acephate	0.45 ± 0.26	20.0
Chloramidophos	0.43 ± 0.32	20.0
Optunal	0.34 ± 0.12	14.7 ± 0.312
Isofenphos-methyl	0.25 ± 0.19	9.67 ± 0.113
Fenamiphos	0.21 ± 0.15	6.51 ± 0.164
Triazophos	0.02 ± 0.002	0[a]
Chlorpyrifos	0.02 ± 0.001	0
Dimethoate	0.03 ± 0.003	0
Methyl-parathion	0.02 ± 0.001	0
Fenitrothion	0.02 ± 0.001	0
Dichlorvos	0.02 ± 0.001	0

[a] Below the level of detection

Cellular location of the MAP-1 methamidophos-degrading enzyme

The methamidophos-degrading activities in medium, periplasmic, cytoplasmic, cell inner membrane and outer membrane fractions of MAP-1 were investigated. It was found that most of the methamidophos-degrading enzyme activity was located on inner membrane (90.4%) and the enzyme activity was 2.36 ± 0.080 μmol min^{-1}. A small quantity of enzyme activity was detected in the periplasm (5.2%) and outer membrane (4.4%) fractions. The results suggest that the MAP-1 methamidophos-degrading enzyme is tightly associated with the cell inner membrane.

Identification of metabolites during methamidophos biodegradation

MS/MS and GC–MS were carried out to identify the metabolites produced during methamidophos biodegradation. In the standard MS (Fig. 2), prominent protonated molecular ions at $m/z = 140$ $[M–H]^-$, $m/z = 141$ $[M–H]^-$, $m/z = 127$ $[M–H]^-$ and $m/z = 111$ $[M–H]^-$ were found, and compounds corresponding to the protonated molecular ions are designated as Product A, B, C and D. However, using GC–MS analysis for further verification, only three compounds with retention times of 7.32, 5.27 and 8.06 min were identified in the total ion chromatography (TIC) profiles (Fig. 2, inset). All of the products indentified by GC–MS were in agreement with the results from MS/MS (Fig. 3).

Product A: the m/z was 140 $[M–H]^-$ in standard MS with the characteristic second-order MS fragment ion peaks at $m/z = 125$ ($[M–H]^-$–CH_3), 93

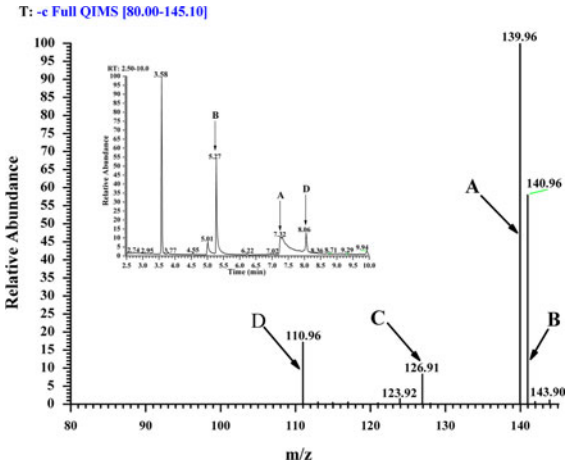

Fig. 2 Standard mass spectrum and total ion chromatography profiles (*inset*) for methamidophos and its metabolites produced during methamidophos degradation by MAP-1. In the standard mass spectrum, peaks with prominent protonated molecular ions at $m/z = 141$ $[M–H]^-$, $m/z = 140$ $[M–H]^-$, $m/z = 127$ $[M–H]^-$ and $m/z = 111$ $[M–H]^-$ are marked with *letters* A to D, respectively. In the total ion chromatography profiles (*inset*), peaks with retention times at 7.32, 5.27 and 8.06 min are marked with letters A, B and D, respectively. The *same letters* indicate the same compounds

($[M–H]^-$–CH_3S), 78 ($[M–H]^-$–CH_3–CH_3S) and 62 ($[M–H]^-$–CH_3–CH_3S–O). Its retention time in TIC profiles was 7.32 min, and its mass-fragmentation pattern was at $m/z = 141(M^+)$, 126 (M^+–CH_3), 111 (M^+–$2CH_3$), 94 (M^+–CH_3S), 79 (M^+–CH_3–CH_3S), 64 (M^+–CH_3–CH_3S–NH), 47 (CH_3S) (Fig. 3, Product A). Thus, it was identified as methamidophos.

Product B showed a prominent protonated molecular ion at $m/z = 141$ $[M–H]^-$ in standard MS (with characteristic fragment ion peaks of second-order MS at $m/z = 126$ ($[M–H]^-$–CH_3), 109 ($[M–H]^-$–CH_3–OH) and 79 ($[M–H]^-$–CH_3–CH_3S) and showed a retention time of 5.27 min in TIC profiles (with the mass-fragmentation pattern at $m/z = 142$ (M^+), 127 (M^+–CH_3), 112 (M^+–CH_3–CH_3), 95 (M^+–CH_3S), 80 (M^+–CH_3–CH_3S), and 64 (M^+–CH_3O–CH_3S)) (Fig. 3, Product B). On the basis of the characteristic fragment ion peaks by MS/MS and the mass-fragmentation pattern by GC–MS, product B was identified as *O,S*-dimethyl hydrogen thiophosphate. It was deduced that the first step of methamidophos degradation by MAP-1 is the cleavage of the P–N bond. During the degradation of methamidophos in MSM-1 supplemented with 0.5% (v/v) methanol, culture samples were collected to detect the production of NH_4^+–N. It was observed that from 24 to 60 h, the culture color changed to red brown when Nessler's Reagent was added (Chen et al. 2009). After 60 h, the culture color returned to the original yellow. These results further indicate that the P–N bond of methamidophos is first broken down to release NH_4^+–N during methamidophos biodegradation.

Product C showed a base peak at $m/z = 127$ $[M–H]^-$, enabling the assignment of the molecular ion $[M^-]$ at $m/z = 128$. The characteristic second-order MS fragment ion peaks at $m/z = 127$ ($[M–H]^-$), 95 ($[M–H]^-$–CH_3–OH), 80 ($[M–H]^-$–CH_3S) and 63 ($[M–H]^-$–CH_3S–OH) are shown in Fig. 3 (Product C). So, Product C was identified as *S*-methyl dihydrogen thiophosphate. It was deduced that the P–O bond of *O,S*-dimethyl hydrogen thiophosphate was cleaved, resulting in the release of the CH_3O group and formation of *S*-methyl dihydrogen thiophosphate.

Product D showed a prominent protonated molecular ion at $m/z = 111$ $[M–H]^-$ in standard MS (with characteristic fragment ion peaks of second order MS at $m/z = 96$ ($[M–H]^-$–CH_3), 80 ($[M–H]^-$–CH_3O) and 63 ($[M–H]^-$–CH_3O–OH) and showed a retention time of 8.01 min in TIC profiles (with the mass-fragmentation pattern at $m/z = 112$ (M^+), 97 (M^+–CH_3), 81 (M^+–CH_3O), 63 (M^+–CH_3O–OH–H) and 48 (M^+–CH_3–OH–O–O) (Fig. 3, Product D). Product D was identified as methyl dihydrogen phosphate, according to the above results. Product D corresponds to the cleavage of the P–S bond of Product B (*O,S*-dimethyl hydrogen thiophosphate) which releases the CH_3S group.

Finally, it is likely that phosphoric acid is formed by the hydrolysis of the P–O bond of Product C (*S*-methyl dihydrogen thiophosphate) to release the CH_3O group or by the hydrolysis of the P–S bond of Product D (methyl dihydrogen phosphate) to free the CH_3S group. During the biodegradation of methamidophos in MSM-2 supplemented with 0.5% (v/v) methanol and 1.0 g l^{-1} NH_4NO_3, culture samples were collected to detect the presence of inorganic phosphorus. It was observed that, from 48 to 120 h, the culture color changed to blue when ammonium molybdate was added (Zhang et al. 2006). After 120 h, the blue color disappeared. These results further prove that methamidophos is ultimately transformed into phosphoric acid.

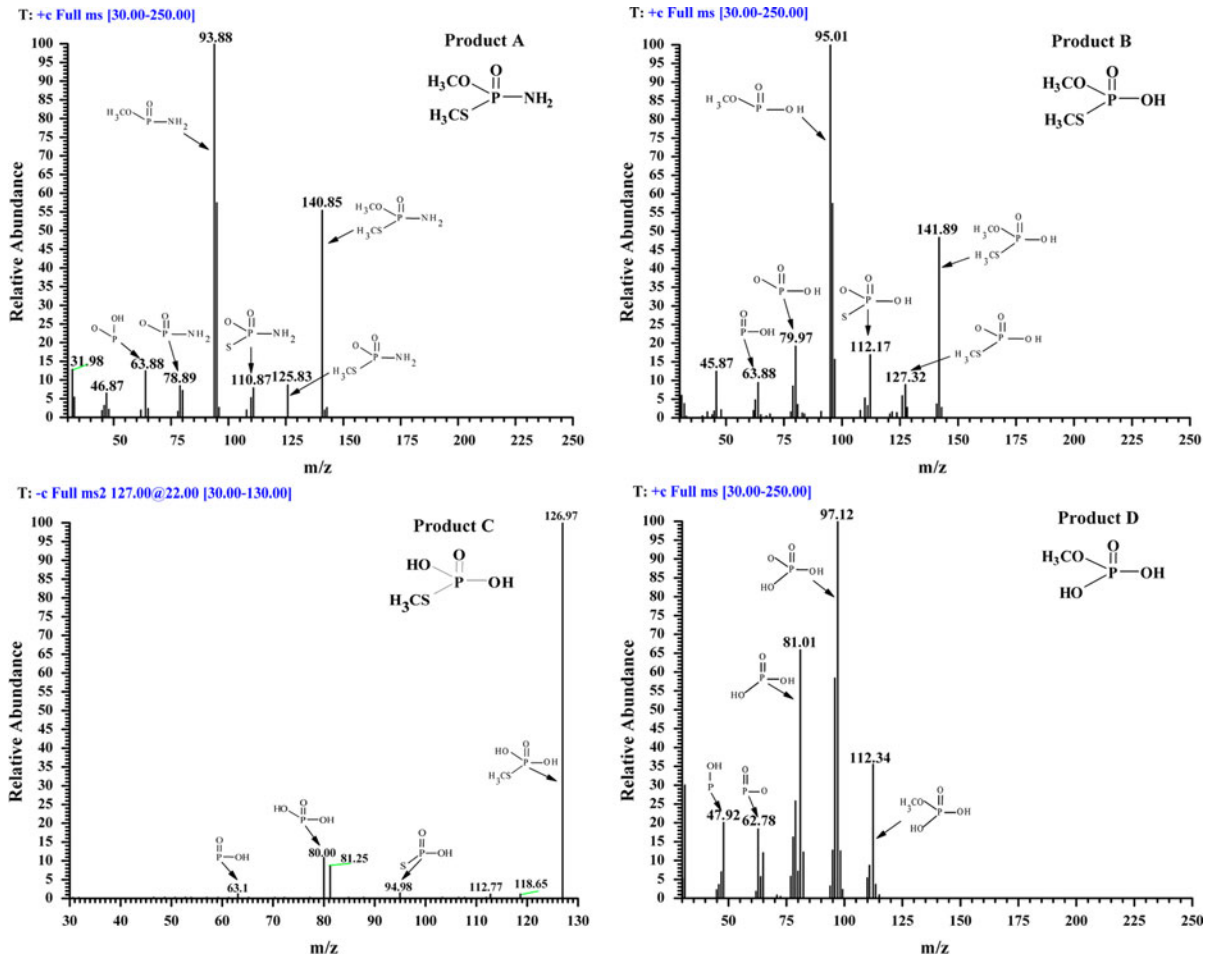

Fig. 3 The GC–MS profile of products A, B and D. Product C was identified by MS/MS. Products A, B, C and D are identified as methamidophos, *O,S*-dimethyl hydrogen thiophosphate, *S*-methyl dihydrogen thiophosphate and methyl dihydrogen phosphate, respectively

Discussion

Microorganisms play key roles in the bioremediation of pesticide-contaminated sites. However, few studies of the microbial degradation of methamidophos have been carried out. To date, only five bacteria (Chao et al. 1999, 2000; Xiao and Wang 1995; Wu et al. 2005; Zheng et al. 2006) and three fungi (Li et al. 1999, 2001; Liu and Zhong 1999) that can degrade methamidophos have been reported. In the present study, a high efficient methamidophos-degrading strain MAP-1 from the genus *Hyphomicrobium* was isolated. Since pure *Hyphomicrobium* cultures were first obtained (Stutzer and Hartleb 1898), they have been characterized as "slow growing organisms" (Attwood and Harder 1972; Hirsch and Conti 1964). These bacteria are usually oligotrophic and sensitive to various common nutrients such as glucose, yeast extract and peptone (Nikitin et al. 1990). *Hyphomicrobium* species are obligately or facultatively methylotrophic strains which can utilize some C_1 substrates, e.g., methanol and formate. *Hyphomicrobium* strains were usually reported for transforming the chlorinated solvents such as dichloromethane (Nikolausz et al. 2005), dimethyl sulfoxide (Takako et al. 2002) and dimethylsulfone (Elena et al. 2000), and also reported for having the ability of denitrification (Karin et al. 1995). To the best of our knowledge, this is the first report of a strain from the genus *Hyphomicrobium* which is capable of degrading methamidophos.

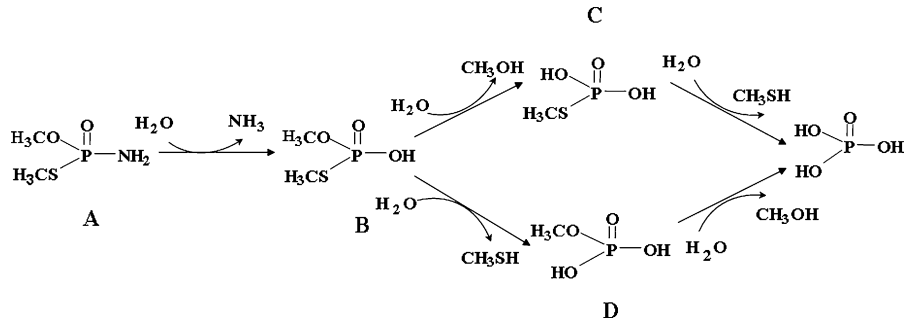

Fig. 4 The proposed degradation pathway of methamidophos by *Hyphomicrobium* sp. MAP-1

Strain MAP-1 was able to utilize methamidophos as its sole carbon, nitrogen and phosphorus source for growth, and it only required 36 h to completely remove 1,000 mg l^{-1} methamidophos in MSM-4 medium under optimal conditions (pH 7.0, 30°C). In addition, it only required 84 h to completely remove 3,000 mg l^{-1} methamidphos. It has been reported that 5 days were needed to remove 83% of 3,000 mg l^{-1} methamidophos by a fungus (Liu and Zhong 1999). Further, our degradation spectrum experiments showed that MAP-1 could efficiently degrade various other organophosphate insecticides such as acephate, chloramidophos, optunal, fenamiphos and isofenphos-methyl. In general, the discovery of MAP-1 has enriched the microorganism sources for the degradation of organophosphate insecticides and is a potentially useful strain for the bioremediation of ecosystems contaminated with high concentrations of methamidophos and/or other organophosphate insecticides.

Due to the simple structure of methamidophos, it was difficult to identify its degradation pathway. It has previously been reported that methamidophos is ultimately mineralized into PO_4^{3-} in aqueous solutions by solar photocatalytic oxidation in the presence of TiO_2 (Malato et al. 1999). In microorganism transformation, *Acinetobacte* sp. HS-A32 can break down the P–N bond of methamidophos to release $–NH_2$ and form NH_4^+ (Zheng et al. 2006). Similarly, using the crude enzyme from *Pseudomonas* sp. S-2 to treat methamidophos, a small quantity of PO_4^{3-} was observed by the ammonium vanadate-molybdate color comparison method (Wu et al. 2005). Finally, a kind of highly active acid phosphatase was identified from *Saccharomyces rouxii* WY-3 by SDS–polyacrylamide gel electrophoresis. This acid phosphatase could break down the methamidophos P–O bond to produce CH_3OH and ultimately transform methamidophos to PO_4^{3-} (Liu et al. 2001). However, in additional to CH_3OH, PO_4^{3-} and NH_4^+, no other metabolites have yet been discovered, and there is still no clear degradation pathway for methamidophos reported in any field.

In this paper, three metabolites (*O,S*-dimethyl hydrogen thiophosphate, *S*-methyl dihydrogen thiophosphate and methyl dihydrogen phosphate) were identified by MS/MS and GC–MS, and the likely MAP-1 methamidophos biochemical degradation pathway was proposed for the first time (Fig. 4). The first step involved in the degradation of methamidophos is the scission of the P–N bond to form *O,S*-dimethyl hydrogen thiophosphate and NH_3. This reaction is identical to those previously reported (Zheng et al. 2006) and was corroborated by: (1) the fact that the color of the culture changed to red brown when Nessler's Reagent was added; and (2) the fact that MAP-1 can also degrade acephate, chloramidophos, optunal, methyl-parathion, fenamiphos and isofenphos-methyl, which all have the P–N bond. *O,S*-dimethyl hydrogen thiophosphate (Product B) was subsequently hydrolyzed at the P–O bond to release $–OCH_3$ and form *S*-methyl dihydrogen thiophosphate (Product C). This reaction is similar with that reported previously (Liu et al. 2001). *O,S*-dimethyl hydrogen thiophosphate could also be hydrolyzed at the P–S bond to release $–SCH_3$ and form methyl dihydrogen phosphate (Product D). It has similarly been reported that methyl dihydrogen phosphate was also produced during the transformation of other organophosphate insecticides (Evgenidou et al. 2006). Likewise, the CH_3SH formation reaction was also found in the hydrolytic degradation of other organophosphorous insecticides (Dannenberg et al. 1998). Finally, *S*-methyl dihydrogen thiophosphate and methyl dihydrogen phosphate were likely transformed into phosphoric acid. This reaction is

implicated by the fact that culture color changed to blue when ammonium molybdate was added (Liu et al. 2001; Malato et al. 1999; Wu et al. 2005). The presented methamidophos degradation pathway in *Hyphomicrobium* sp. MAP-1 offers useful information about the biodegradation mechanism of methamidophos in microorganisms.

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