An Amidase Gene, *ipaH*, Is Responsible for the Initial Step in the Iprodione Degradation Pathway of *Paenarthrobacter* sp. Strain YJN-5

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ABSTRACT Iprodione [3-(3,5-dichlorophenyl) *N*-isopropyl-2,4-dioximidazolidine-1-carboxamide] is a highly effective broad-spectrum dicarboxamide fungicide. Several bacteria with iprodione-degrading capabilities have been reported; however, the enzymes and genes involved in this process have not been characterized. In this study, an iprodione-degrading strain, *Paenarthrobacter* sp. strain YJN-5, was isolated and characterized. Strain YJN-5 degraded iprodione through the typical pathway, with hydrolysis of its N-1 amide bond to *N*-(3,5-dichlorophenyl)-2,4-dioximidazolidine as the initial step. The *ipaH* gene, encoding a novel amidase responsible for this step, was cloned from strain YJN-5 by the shotgun method. IpaH shares the highest similarity (40%) with an indoleacetamide hydrolase (IAHH) from *Bradyrhizobium diazoefficiens* USDA 110. IpaH displayed maximal enzymatic activity at 35°C and pH 7.5, and it was not a metalloamidase. The k_{cat} and K_m of IpaH against iprodione were 22.42 s⁻¹ and 7.33 μM, respectively, and the catalytic efficiency value (k_{cat}/K_m) was 3.09 μM⁻¹ s⁻¹. IpaH has a Ser-Ser-Lys motif, which is conserved among members of the amidase signature family. The replacement of Lys82, Ser157, and Ser181 with alanine in IpaH led to the complete loss of enzymatic activity. Furthermore, strain YJN-5M lost the ability to degrade iprodione, suggesting that *ipaH* is the only gene responsible for the initial iprodione degradation step. The *ipaH* gene could also be amplified from another previously reported iprodione-degrading strain, *Microbacterium* sp. strain YJN-G. The sequence similarity between the two IpaHs at the amino acid level was 98%, indicating that conservation of IpaH exists in different strains.

IMPORTANCE Iprodione is a widely used dicarboxamide fungicide, and its residue has been frequently detected in the environment. The U.S. Environmental Protection Agency has classified iprodione as moderately toxic to small animals and a probable carcinogen to humans. Bacterial degradation of iprodione has been widely investigated. Previous studies demonstrate that hydrolysis of its N-1 amide bond is the initial step in the typical bacterial degradation pathway of iprodione; however, enzymes or genes involved in iprodione degradation have yet to be reported. In this study, a novel *ipaH* gene encoding an amidase responsible for the initial degradation step of iprodione in *Paenarthrobacter* sp. strain YJN-5 was cloned. In addition, the characteristics and key amino acid sites of IpaH were investigated. These findings enhance our understanding of the microbial degradation mechanism of iprodione.

KEYWORDS *Paenarthrobacter* sp. strain YJN-5, iprodione, amidase, initial degradation

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Iprodione, a kind of dicarboxamide fungicide, is widely used to control fungal pathogens in crops. Although iprodione is not chemically stable because of its hydrolysis in the environment, its relatively low soil absorption coefficient (K_{oc} ; 400 ml g⁻¹) results in high soil mobility (1). Iprodione and its metabolites have been detected in drainage and surface waters (2, 3). The U.S. Environmental Protection Agency (USEPA) has classified iprodione as moderately toxic to small animals and a probable carcinogen to humans (4, 5); therefore, great concern and interest have been raised regarding its environmental behavior and degradation mechanism.

Although chemical alkaline hydrolysis causes the dissipation of iprodione (6, 7), some studies demonstrate that biodegradation also plays an important role in this process. To date, several bacteria capable of degrading iprodione have been isolated and characterized, including *Arthrobacter* sp. strain MA6, *Arthrobacter* sp. strain C1, *Achromobacter* sp. strain C2, *Pseudomonas* sp. strain 2, *Pseudomonas* sp. strain 3, *Microbacterium* sp. strain YJN-G, and *Microbacterium* sp. strain CQH-1 (8–13). The degradation pathway of iprodione has been proposed and summarized based on intermediate metabolites produced during its degradation by different strains (see Fig. S1 in the supplemental material). During the degradation process, hydrolysis of the N-1 amide bond is the common initial step. However, because the evidence for iprodione degradation comes from metabolite identification, the molecular basis of the pathway has not been described.

In this study, we focus on the isolation of iprodione-degrading strains and cloning of the amidase gene, *ipaH*, which is responsible for the hydrolysis at the N-1 amide bond, as it is the common initial step in the degradation of iprodione. Additionally, the characteristics of IpaH and the abundance of *ipaH* were investigated. Results of the present study suggest that the *ipaH* gene is crucial for the degradation of iprodione, thereby furthering our understanding of the iprodione degradation mechanism.

RESULTS

Isolation and identification of an iprodione-degrading strain. An iprodione-degrading bacterial strain, YJN-5, was isolated that can use iprodione as the sole carbon source for growth. This strain formed a clear transparent halo on MSM plates amended with 0.6 mM iprodione (see Fig. S2 in the supplemental material). It is a rod-shaped, Gram-positive bacterium with dimensions of 0.8 to 1.2 μ m by 1.8 to 4.0 μ m. Strain YJN-5 can hydrolyze citric acid, propionic acid, formic acid, and uric acid but not benzoic acid, adipic acid, or malonic acid. It can utilize L-asparagine, D-glucose, D-xylose, 4-aminobutyrate, and *p*-hydroxybenzoate but not L-histidine or butanediol. These characteristics are consistent with the general properties of *Paenarthrobacter* species (14). The 16S rRNA gene sequence of YJN-5 is highly similar to those of known *Paenarthrobacter* strains, including *P. ureafaciens* DSM201626^T (99.65%), *P. nicotovorans* DSM420^T (99.24%), and *P. histidinovorans* DSM20115^T (98.89%). A phylogenetic tree constructed on the basis of 16S rRNA gene sequences of strain YJN-5 and its close relatives is presented in Fig. S3. Based on the above-described information, strain YJN-5 was preliminarily identified as *Paenarthrobacter* species.

Degradation of iprodione by strain YJN-5. The degradation kinetics of iprodione and growth of YJN-5 were simultaneously investigated (Fig. 1). During the first 10 h, strain YJN-5 underwent a lag phase and did not show obvious growth, and there was a slight decrease in conjunction with the decrease in iprodione, suggesting that initial degradation is a rapid step, like hydrolysis. Strain YJN-5 then degraded the intermediate metabolites further and used them as a carbon source for growth, at the same time continuing *de novo* degradation of iprodione. After 80 h of incubation, 1.5 mM iprodione was almost completely depleted (95%), and the cell density had increased from 1.18×10^7 CFU ml⁻¹ to 2.25×10^7 CFU ml⁻¹, indicating that YJN-5 can utilize iprodione to support its growth.

Analysis and identification of metabolites of iprodione. For the sample collected 6 h after inoculation, four compounds (compounds I, II, III, and IV) were detected by high-performance liquid chromatography (HPLC) (Fig. 2A), with retention times of 9.68

FIG 1 Utilization of iprodione as the sole carbon source for growth by *Paenarthrobacter* sp. strain YJN-5. ▽, iprodione control; ▼, iprodione with strain YJN-5; ○, cell density of strain YJN-5 without iprodione; ●, cell density of strain YJN-5 with iprodione; ■, 3,5-dichloroaniline. Cell growth was determined by the colony counting method. Error bars represent the standard errors from three replicates.

min, 4.79 min, 4.40 min, and 7.86 min. The prominent protonated molecular ion of compound I was m/z 330.0411 $[M+H]^+$, which was identified as iprodione ($C_{13}H_{14}Cl_2N_3O_3^+$, m/z 330.0407), with a 1.2-ppm error (Fig. 2B). The molecular ion mass of compound II was m/z 244.9878 $[M+H]^+$, which is consistent with the protonated derivative of *N*-(3,5-dichlorophenyl)-2,4-dioxoimidazolidine ($C_9H_7Cl_2N_2O_2^+$, m/z 244.9879), with a −0.5-ppm error (Fig. 2C). The molecular ion mass of compound III was m/z 262.9986 $[M+H]^+$, which corresponds to the protonated derivative of (3,5-dichlorophenylurea) acetic acid ($C_9H_6Cl_2N_2O_3^+$, m/z 262.9985), with a 0.9-ppm error (Fig. 2D). The molecular ion mass of compound IV was m/z 161.9869 $[M+H]^+$, which is in agreement with the protonated derivative of 3,5-dichloroaniline ($C_6H_6Cl_2N^+$, m/z 161.9872), with a −1.8-ppm error (Fig. 2E). Generally, a mass error between −5 ppm and 5 ppm is acceptable for the identification of compounds (15). Therefore, a putative degradation pathway of iprodione in strain YJN-5 was proposed based on the results of this study and previous reports (8–13, 16) (Fig. 2F). This pathway is the same as the typical one (Fig. S1). The initial step is hydrolysis of the N-1 amide bond of iprodione to *N*-(3,5-dichlorophenyl)-2,4-dioxoimidazolidine and a putative metabolite isopropylcarbamic acid, and this compound might be converted to isopropylamine and CO_2 by spontaneous hydrolysis; however, neither isopropylcarbamic acid nor isopropylamine was directly detected by HPLC, but isopropylamine was detected in the reported degradation pathway of iprodione (9–11). Also, isopropylamine can be further degraded by strain YJN-5, as it is able to grow with isopropylamine as the sole carbon source (data not shown). In addition, the hydrolysis of (3,5-dichlorophenylurea) acetic acid (compound III) resulted in glycine, CO_2 , and 3,5-dichloroaniline as the end product, and it would accumulate in the culture (Fig. 1 and 2). Glycine can also act as an additional carbon source for the growth of strain YJN-5 (data not shown). This may explain why strain YJN-5 cannot completely degrade iprodione but can utilize iprodione as the sole carbon source for growth.

Cloning and sequence analysis of the *ipaH* gene. The shotgun method was used to construct the genomic library of strain YJN-5, and formation of a clear transparent halo around the colony on LB plates containing 100 mg liter^{−1} ampicillin and 0.6 mM iprodione was used as a marker for identifying positive clones. Two positive clones, designated *E. coli*-pR1 and *E. coli*-pR2, were selected from approximately 12,000 transformants, and their ability to degrade iprodione was further confirmed by HPLC and tandem mass spectrometry (MS/MS). The results showed that both of the positive clones could transform iprodione to *N*-(3,5-dichlorophenyl)-2,4-dioxoimidazolidine (Fig. S4), and this was consistent with the initial hydrolysis step of iprodione in strain YJN-5.

FIG 2 Degradation pathway of iprodione in strain YJN-5. (A) HPLC analysis of metabolites that appeared during the degradation of iprodione by strain YJN-5. (B) MS/MS analysis of compound I (m/z 330.0411 $[M+H]^+$), which was identified as iprodione. (C) MS/MS analysis of compound II (m/z 244.9878 $[M+H]^+$), which was identified as *N*-(3,5-dichlorophenyl)-2,4-dioximidazolidine. (D) MS/MS analysis of compound III (m/z 262.9986 $[M+H]^+$), which was identified as (3,5-dichlorophenylurea) acetic acid. (E) MS/MS analysis of compound IV (m/z 161.9869 $[M+H]^+$), which was identified as 3,5-dichloroaniline. (F) The metabolic pathway of iprodione degradation in strain YJN-5.

The recombinant plasmids harbored by *E. coli*-pR1 and *E. coli*-pR2 were designated pR1 and pR2, respectively (Fig. S4A). They carried 5,716-bp and 7,828-bp inserts, respectively, which could be fused into a 10,850-bp fragment with 2,694-bp overlap. The physical map of this fragment is shown in Fig. S5. Computational analysis of its sequence by online ORF Finder and BLASTx (www.ncbi.nlm.nih.gov) identified six complete open reading frames (ORFs), designated *tpnA*, *orf1*, *orf2*, *orf3*, *ipaH*, and *orf4*. The protein sequences encoded by these ORFs were used as queries in a BLASTP search (UniProtKB/Swiss-Port database), and functions were proposed for each ORF (Table S1). TnpA exhibits 38% similarity to a transposase of transposon Tn3926 (P13694) from *Escherichia coli* (17). *orf1* encodes a protein consisting of 296 amino acid residues that shows moderate sequence identity with an RNA polymerase sigma factor, SigA (P77994), from strain *Thermotoga maritima* MSB8 (18), while *orf2* encodes a protein with 30% similarity to a nucleotidyltransferase (Q7U911) from *Synechococcus* sp. strain WH 8102 (19). *orf3* is located 251 bp downstream of *ipaH*, and its deduced amino acid sequence is similar to that of an NAD-dependent dihydropyrimidine dehydrogenase (O33064) from *Mycobacterium leprae* strain TN (20). *orf4* encodes a helicase, which shows the highest sequence identity to a chromodomain-helicase-DNA-binding protein 1-like (Q7ZU90).

The *ipaH* gene is 1,410 bp long and encodes a hydrolase of 469 amino acids. The deduced IpaH protein shares a low amino acid sequence identity (27 to 40%) with several biochemically characterized amidases from *Bradyrhizobium diazoefficiens* USDA 110 (indole-3-acetamide hydrolase, IAAH [P59385], 40% identity) (21), *Bradyrhizobium japonicum* (indole-3-acetamide hydrolase, IAAH [P19922], 39% identity) (22), *Paracoccus huijuniae* FLN-7 (arylamides, AmpA [JQ388838], 30% identity) (23), *Acinetobacter* sp. strain Ooi24 (*N*-acyl-L-homoserine lactones acylase, AmiE [AB933638], 28% identity) (24), and *Thermus thermophilus* HB8 (glutamyl-tRNA amidotransferase subunit A, GatA [Q9LCX3], 27% identity) (25). These five enzymes belong to the amidase signature (AS) family, which is characterized by a conserved domain containing a signature GGSSGG motif (21, 26). Moreover, a highly conserved catalytic triad of the amidase family, the Ser-Ser-Lys (Ser157, Ser181, and Lys82) motif, was identified in IpaH and these five enzymes (Fig. S6). These results suggest that IpaH can be classified as a member of the amidase signature family (27). Considering that iprodione has an N-1 amide bond, IpaH may be the enzyme responsible for hydrolyzing this bond. Furthermore, based on sequence alignment and phylogenetic analysis, IpaH shares low similarity ($\leq 40\%$) with amidase signature sequences and other biochemically characterized amidases (available from the NCBI Swiss-Prot protein database) (Fig. S7). Proteins in the amidase signature family were grouped into IAAH, GatA, fatty acid amide hydrolase (FAAH), and peptide amidase (PMD). IpaH was on a branch with IAAH 1, indicating a closer relationship with proteins in that group. IpaH also forms an independent lineage with characterized amidases, including IAAH from *Bradyrhizobium* (39 to 40% identity), AmpA from *Paracoccus* species (30% identity), and AmiE from *Acinetobacter* species (28% identity), suggesting an evolutionarily close relationship. These results indicate that IpaH constitutes a novel amidase within the amidase signature family.

Heterogeneous gene expression of *ipaH*. To further investigate if *ipaH* is responsible for hydrolyzing the N-1 amide bond of iprodione in strain YJN-5, the *ipaH* gene was cloned and expressed in *E. coli* BL21(DE3). The yield of purified IpaH was estimated to be approximately 17.8 ± 2.8 mg per liter of culture. The molecular mass of the native IpaH was calculated to be 62 kDa by gel filtration chromatography; purified IpaH appeared as a single band on SDS-PAGE, with a molecular mass of ~ 50 kDa. Thus, we deduced that IpaH exists naturally as a monomer (Fig. S8). The enzyme assay of IpaH showed that it hydrolyzed the N-1 amide bond of iprodione, producing *N*-(3,5-dichlorophenyl)-2,4-dioximidazolidine (compound II) (Fig. 3), which was the only product detected in the assay. Therefore, *ipaH* is responsible for the hydrolysis of the N-1 amide bond of iprodione, which is the initial step of iprodione degradation in strain YJN-5.

FIG 3 Identification of hydrolysis product of iprodione by IpaH. (A) HPLC analysis of the hydrolysis product of iprodione by IpaH. (B) MS/MS analysis of compound I (m/z 330.0407 [$M+H$] $^+$), which was identified as iprodione. (C) MS/MS analysis of compound II (m/z 244.9879 [$M+H$] $^+$), which was identified as *N*-(3,5-dichlorophenyl)-2,4-dioxoimidazolidine.

Biochemical characterization of purified IpaH. The specific activity of IpaH is 20.88 U mg^{-1} for iprodione, while the V_{max} , K_m , and k_{cat} of IpaH for iprodione are 0.42 ± 0.01 $\mu mol\ s^{-1}\ mg^{-1}$, 7.33 ± 0.17 μM , and $22.42 \pm 0.82\ s^{-1}$, respectively (Fig. S9). Optimal IpaH activity was observed at pH 7.5 and 35°C. The enzyme was relatively stable up to 50°C, as it retained more than 60% of its activity at 50°C for 1 h; however, at a higher temperature of 70°C, residual IpaH activity fell below 10% in 1 h. IpaH exerted high levels of activity at pH 7.0 to 9.0 and retained 85% of its activity after storage at pH 6.0 to 9.0 for 2 h (Fig. S10). The activity of IpaH was not affected by EDTA in the tested concentrations, indicating that the enzyme is not a metalloamidase.

Substrate spectrum of IpaH. IpaH exhibited a preference for iprodione over any other substrate tested. As for the other substrates, IpaH was highly active against several aromatic secondary amine compounds, including propanil, 4-nitroacetanilide, and leucine-*para*-nitroanilide, with the relative enzyme activity being 71.6%, 53.8%, and 45.3%, respectively. IpaH showed low activity against primary amine compounds, including aromatic and short-chain aliphatic amide compounds, such as benzamide, acetamide, propanamide, and urea. IpaH also showed low activity toward amino acids, L-glutamine (4.5%), and L-asparagine (3.6%). Furthermore, IpaH showed no activity against indole-3-acetamide, chlorpropham, carbendazim, linuron, *N*-isopropylacetamide, and *N*-acetyl glycine (Table S2).

The *ipaH* gene is essential for the degradation of iprodione. To verify whether *ipaH* is the only gene involved in the initial degradation step of iprodione by strain

FIG 4 Transparent halos formed by tested strains on LB agar supplemented with 0.6 mM iprodione.

YJN-5, *ipaH* was disrupted through a single-crossover event. The resulting mutant strain, YJN-5M, lost the ability to hydrolyze iprodione. The complemented strain YJN-5M(pBBR1-*ipaH*) regained the ability to degrade iprodione (Fig. S11) and formed a transparent halo on LB agar supplemented with 0.6 mM iprodione (Fig. 4). Strain YJN-5M(pBBR1-*ipaH*) not only regained the ability to hydrolyze iprodione to compound II but also further degraded compound II to compound III and the end product compound IV, which is the same as that for strain YJN-5. As the above-mentioned two positive clones (*E coli*-pR1 and *E coli*-pR2) could only transform iprodione to compound II (Fig. S5), there are no other subsequent genes related to the degradation of iprodione on either side of *ipaH* in the 10,850-bp DNA fragment. Taken together, these results show that *ipaH* is essential for the degradation of iprodione by strain YJN-5.

Interestingly, the *ipaH* gene can be amplified from another iprodione-degrading strain, *Microbacterium* sp. strain YJN-G, previously isolated by our laboratory (12), with primers ipaH-A and ipaH-B. The initial iprodione degradation step in strain YJN-G was also the hydrolysis of its N-1 amide bond. Moreover, the amino acid sequence similarity between the two IpaH proteins is 98% (Fig. S6), indicating conservation of IpaH sequences in these two strains. The V_{max} , K_m , and k_{cat} of IpaH from strain YJN-G for iprodione are $0.45 \pm 0.02 \mu\text{mol s}^{-1} \text{mg}^{-1}$, $8.90 \pm 1.47 \mu\text{M}$, and $16.15 \pm 1.53 \text{ s}^{-1}$, respectively (Fig. S12).

The conserved amino sites of IpaH. The conserved Ser-Ser-Lys motif (Lys82, Ser157, and Ser181) of IpaH was replaced by alanine, resulting in three variants (IpaH-K82A, IpaH-S157A, and IpaH-S181A) (Fig. S13), and the enzyme activity assay shows that all of the variants lost hydrolysis activity against iprodione. These results further prove that IpaH is an amidase signature enzyme containing the highly conserved catalytic triad Ser-Ser-Lys.

DISCUSSION

To date, several iprodione-degrading bacteria have been reported from the genera *Arthrobacter*, *Achromobacter*, *Pseudomonas*, and *Microbacterium* (8–13). Strain YJN-5 is an iprodione-degrading strain identified from the genus *Paenarthrobacter* species, thereby enriching the diversity of iprodione degraders in the environment. Among the reported iprodione-degrading strains, *Arthrobacter* sp. strain MA6 degraded 8.8 μM iprodione to approximately 0.5 μM within 37 h (8). The coculture of *Pseudomonas* sp. strain 3 and *Pseudomonas paucimobilis* strain 4 was able to completely transform 80 μM iprodione within approximately 10 h (11). *Arthrobacter* sp. strain C1 was able to degrade 60 μM iprodione within 8 h (10), while *Microbacterium* sp. strain CQH-1 degraded 0.3 mM iprodione within 96 h (13). Previously, our laboratory reported that *Microbacterium* sp. strain YJN-G can degrade 0.15 mM iprodione within 20 h (12). In this study, strain YJN-5 was capable of degrading 1.5 mM iprodione within 80 h.

Strain YJN-5 degrades iprodione through three successive hydrolysis steps, resulting in the end product 3,5-dichloroaniline (Fig. 2F), which is the same as the typical pathway (see Fig. S1 in the supplemental material). In our study, *N*-(3,5-dichlorophenyl)-2,4-dioximidazolidine (compound II) was the only metabolite detected during the initial degradation step in strain YJN-5 (Fig. 2F), and it was also the only metabolite

detected in the transformation of iprodione by the two positive clones as well as in the enzyme assay of IpaH (Fig. 3A and Fig. S4). According to our results and previous reports on the degradation pathway of iprodione (9–11), we concluded that hydrolysis of the N-1 amide bond to *N*-(3,5-dichlorophenyl)-2,4-dioximidazolidine is the initial step in the degradation pathway (Fig. 3B and C).

IpaH is responsible for the initial step of iprodione degradation in strain YJN-5, and it is an amidase with a conserved Ser-Ser-Lys motif. The substitution of Ser-Ser-Lys for alanine leads to complete loss of enzymatic activity, which is consistent with the corresponding amidase variants in *Paracoccus* sp. strain M-1 (PamH) and other amidases containing the Ser-Ser-Lys triad, which also lost amidase activity (27–29). Although there are 7 amino acid differences between the IpaHs from strains YJN-5 and YJN-G, they both have the conserved active catalytic triad Ser-Ser-Lys. However, IpaH in strain YJN-5 shows higher catalytic efficiency (1.39-fold increase in k_{cat}/K_m), and we will further study the role of these 7 amino acids in causing this difference.

Of the tested substrates, IpaH of strain YJN-5 showed relatively high activity against several aromatic secondary amine compounds, including propanil, 4-nitroacetanilide, and leucine-*para*-nitroanilide, and weak activity toward short-chain primary amine aliphatic amide compounds and amino acid amides, including benzamide, acetamide, propanamide, urea, L-glutamine, and L-asparagine, which was similar to the substrate specificity of the acylamidase from *Rhodococcus erythropolis* TA37, which could hydrolyze certain *N*-substituted aromatic amides (30). Furthermore, IpaH had no activity against *N*-isopropylacetamide and *N*-acetyl glycine. Interestingly, although IpaH shows the highest similarity (40%) to an amidase signature protein (IAAH) that is capable of converting indole-3-acetamide to indole-3-acetic acid (31), it does not exhibit activity against indole-3-acetamide. Also, IpaH has different substrate specificities from those of some other amidases, such as AmpA (23) and PamH (28), which can hydrolyze some pesticides with amide bonds. IpaH, AmpA, and PamH all belong to the amidase signature (AS) enzyme family with a Ser-Ser-Lys motif and can hydrolyze propanil; however, AmpA can hydrolyze chlorpropham while IpaH cannot. Interestingly, IpaH hydrolyzed 4-nitroacetanilide but PamH could not. Additionally, IpaH did not show activity against carbendazim and linuron, although they are both aromatic secondary amino compounds. In summary, IpaH amidase prefers aromatic secondary amino compounds to aliphatic amide compounds. Nevertheless, it has a narrow substrate spectrum. Therefore, the high catalyzing efficiency of IpaH to hydrolyze iprodione suggests that *ipaH* has evolved to take part in this specific catabolic pathway; however, this hypothesis requires further study.

MATERIALS AND METHODS

Chemicals and media. Iprodione (purity 97%) was purchased from Sigma-Aldrich Chemical Co. (Shanghai, China). *N*-(3,5-dichlorophenyl)-2,4-dioximidazolidine (98.5%), 3,5-dichloroaniline (98%), indole-3-acetamide (98%), propanil (99%), 4-nitroacetanilide (98%), leucine-*para*-nitroanilide (99%), chlorpropham (98%), carbendazim (98%), linuron (98%), benzamide (99%), *N*-isopropylacetamide (99%), *N*-acetyl glycine (99%), L-glutamine (98.5%), L-asparagine (99%), acetamide (98.5%), propanamide (96%), and urea (99%) were purchased from J&K Scientific Ltd. (Shanghai, China). Luria-Bertani (LB) broth consisted of the following components (in g liter⁻¹): 10.0 tryptone, 5.0 yeast extract, and 10.0 NaCl. Mineral salts medium (MSM) consisted of the following components (in g liter⁻¹): 1.0 NH₄NO₃, 1.0 NaCl, 1.5 K₂HPO₄, 0.5 KH₂PO₄, 0.2 MgSO₄ · 7 H₂O, pH 7.0; the iprodione mineral salts medium (IMM) was MSM supplemented with 0.15 mM iprodione unless otherwise stated. Because strain YJN-5 cannot use acetone as a carbon source for growth, the iprodione stock solutions (10,000 mg liter⁻¹) were prepared in acetone and sterilized by membrane filtration with a pore size of 0.22 μm. All other chemical reagents were of the highest analytical purity.

Strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37°C in LB broth on a rotary shaker (180 rpm) or on LB agar (1.5%, wt/vol) plates. Strain YJN-5, deposited in the China Center for Type Culture Collection (deposition number CCTCC M 2017440), was grown aerobically in LB broth or IMM at 30°C unless otherwise stated.

Isolation and identification of iprodione-degrading strains. Soil samples were collected from iprodione-applied vineyards in Hebei province, China. Approximately 5.0 g of soil was placed in a 250-ml Erlenmeyer flask containing 50 ml of IMM. The culture was incubated at 30°C on a rotary shaker (180 rpm) for approximately 4 days. Five milliliters of the enrichment culture was subcultured into fresh IMM every 4 days. Iprodione-degrading strains were isolated by spreading serially diluted enrichment cultures on

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s) ^a	Source or reference
<i>Paenarthrobacter</i> sp. strain YJN-5	Degrades iprodione, Str ^r	This study
<i>Microbacterium</i>		
YJN-G	Degrades iprodione	12
YJN-5M	<i>ipaH</i> insertion mutant of <i>Paenarthrobacter</i> sp. strain YJN-5, Str ^r , Gm ^r	This study
YJN-5M(pBBR1- <i>ipaH</i>)	<i>ipaH</i> gene complemented by pBBR1- <i>ipaH</i> in YJN-5M, Str ^r , Gm ^r , Km ^r	This study
<i>E. coli</i>		
DH5α	F [−] <i>recA1 endA1 thi-1 supE44 relA1 deoR Δ(lacZYA-argF)U169 φ80dlacZΔM15</i>	Vazyme
BL21(DE3)	F [−] <i>ompT hsdS_B(r_B[−] m_B[−]) dcm gal λ(DE3)</i>	Vazyme
Plasmids		
pMD19-T	TA clone vector, Amp ^r	TaKaRa
pUC118 BamHI/BAP	DNA library construction vector, Amp ^r	TaKaRa
pR1	pUC118 derivative carrying 5,716-bp insertion including <i>ipaH</i> gene, Amp ^r	This study
pR2	pUC118 derivative carrying 7,828-bp insertion including <i>ipaH</i> gene, Amp ^r	This study
pET-29a(+)	Expression vector, Km ^r	Laboratory stock
pET- <i>ipaH</i>	pET-29a(+) derivative carrying <i>ipaH</i> , Km ^r	This study
pEX18Gm	Gene knockout vector, <i>oriT</i> , <i>sacB</i> , Gm ^r	42
pEX- <i>ipaH</i>	<i>ipaH</i> gene knockout vector containing partial homologous regions of <i>ipaH</i> , Gm ^r	This study
pBBR1MCS-2	Broad-host-range vector, Km ^r	44
pBBR1- <i>ipaH</i>	<i>ipaH</i> gene complementation vector containing <i>ipaH</i> , Km ^r	This study
pET-K82A	pET-29a(+) derivative carrying <i>ipaH</i> -K82A, Km ^r	This study
pET-S157A	pET-29a(+) derivative carrying <i>ipaH</i> -S157A, Km ^r	This study
pET-S181A	pET-29a(+) derivative carrying <i>ipaH</i> -S181A, Km ^r	This study

^aStr^r, streptomycin resistant; Gm^r, gentamicin resistant; Amp^r, ampicillin resistant; Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant.

MSM plates (1.5% [wt/vol] purified agar) containing 0.6 mM iprodione as the sole carbon source. After 4 rounds of enrichment, an iprodione-degrading strain, YJN-5, was obtained and used for further studies. HPLC was used to determine the concentration of iprodione and confirm degradation following strain isolation.

Identification of strain YJN-5 was performed according to *Bergey's Manual of Determinative Bacteriology* (32) and by sequence analysis of the 16S rRNA gene. Genomic DNA was extracted by the high-salt-concentration precipitation method (33). The 16S rRNA gene was amplified by PCR using standard procedures (34). The PCR product was ligated into the pMD19-T vector (TaKaRa Biotechnology, Dalian, China) and then transformed into *E. coli* DH5α. Inserts of the recombinant plasmid harbored by positive clones were sequenced by Nanjing GeneScript Biotechnology Co., Ltd. (Nanjing, China). The 16S rRNA gene sequence (1,487 bp) was deposited in GenBank, and sequence homology was determined by comparisons to available sequences using the EzTaxon-e server (35). Phylogenetic analysis was performed using the software package MEGA 6.0 after multiple alignment of the data by CLUSTALX (36). A phylogenetic tree was built by the neighbor-joining method, and evolutionary distances were calculated according to Kimura's two-parameter model (37). In each case, bootstrap values were calculated based on 1,000 replicates (38).

Degradation of iprodione by strain YJN-5. Cells of strain YJN-5 were cultured in liquid LB medium for 12 h at 30°C and then collected by centrifugation at 6,000 × *g* for 5 min. The cell pellets were washed twice with sterilized MSM, adjusted to an optical density at 600 nm (OD₆₀₀) of approximately 1.5, and used as the inoculant. An aliquot of the cells (2%, vol/vol) was inoculated into a 100-ml Erlenmeyer flask containing 20 ml of MSM supplemented with 1.5 mM iprodione as the sole source of carbon. The flasks were then incubated at 30°C with shaking (180 rpm).

At each sampling point, six flasks were sacrificed for various measurements. Three flasks were used to measure the iprodione concentration or for identification of metabolites by HPLC or MS/MS, while three flasks were used to determine the growth of strain YJN-5 by colony counting. Each treatment was performed in triplicate, and control experiments (medium without inoculum) were carried out under the same conditions.

Cloning of the iprodione-hydrolyzing *ipaH* gene. The shotgun method was used to clone the iprodione-hydrolyzing gene, as outlined below. Genomic DNA of strain YJN-5 was extracted by the high-salt-concentration precipitation method (33). The genomic DNA library of strain YJN-5 was constructed as describe by Wang et al. (39). The 2- to 6-kb fragments were recovered with the DNA gel extraction kit (Omega Bio-Tek Biotechnology Ltd., USA) and ligated into pUC118 (BamHI/BAP) plasmid. The ligation product was transformed into competent *E. coli* DH5α cells, which were then plated on LB plates containing 100 mg liter^{−1} ampicillin and 0.6 mM iprodione. The plates were incubated at 37°C for 12 h and then at 10°C for 48 h. Positive clones that produced clear transparent halos indicative of iprodione hydrolysis were selected and further tested by HPLC and MS/MS analysis for their ability to hydrolyze iprodione. The inserted fragments in the recombinant plasmids harbored by the confirmed positive clones were sequenced by Nanjing GeneScript Biotechnology Co., Ltd. (Nanjing, China). BLASTN and BLASTP (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) were used to analyze the nucleotide sequences and to deduce the amino acids, respectively.

TABLE 2 Primers used in this study

Primer	Sequence (5'–3')	Purpose
27F 1492R	AGAGTTTGATCCTGGCTCAG TACGGCTACCTTGTTACGACTT	To amplify the 16S ribosomal RNA gene
IH-F IH-R	TAAGAAGGAGATATACATATGTCAGATCAGTTGTGGTCAAAGAGTG GTGGTGGTGGTGGTCTCGAGACCAGCGTTGATGAACGGC	Construction of plasmid pET- <i>ipaH</i>
KA-F KA-R	TATGACCATGATTACGAATCTTGGGGGAAGGCGCCATA CAGGTCGACTCTAGAGGATCCTCAGTAGCCAGCCGCGGC	Construction of plasmid pEX- <i>ipaH</i>
KT-F KT-R	TCCGACCCAGCGGGAGACC GCCGTGCGAGTCAGATGGA	Detect integrated sequence in strain YJN-5M
CP-F CP-R	GATAAGCTTGATATCGAATCCGCGATGAGAAAGCAGAAATG CGCTCTAGAACTAGTGGATCCCTAACCAGCGTTGATGAACGG	Construction of plasmid pBBR1- <i>ipaH</i>
<i>ipaH</i> -A <i>ipaH</i> -B	ATGTCAGATCAGTTGTGGTCAAAGAGTGCTA CTAACCAGCGTTGATGAACGGCGAGA	Amplify <i>ipaH</i> gene
K82A-F K82A-R	CCCGATCACCTCGCGGTGAATATTGACCTCGTCGGT CAATATTCACCGCGAGGGTGATCGGGACGC	Amplify mutant gene <i>ipaH</i> -K82A with Lys82 replaced Ala82
S157A-F S157A-R	GCTGGCGGAGCGTCAGGCGGCGAG GCCTGACGCTCCGCCAGCCGTGC	Amplify mutant gene <i>ipaH</i> -S157A with Ser157 replaced to Ala157
S181A-F S181A-R	GATCTCGTTGGTGCCTGCGGAATCCTGCG GATTCCGACGCGACCAACGAGATCATTCCCGAC	Amplify mutant gene <i>ipaH</i> -S181A with Ser181 replaced to Ala181

Gene expression and purification of the recombinant enzyme. The *ipaH* gene was amplified from the genomic DNA of strain YJN-5 using primers IH-F and IH-R (Table 2). The resulting amplicon was ligated into the NdeI- and XhoI-digested pET-29a(+) with the ClonExpress II one-step cloning kit (Vazyme Biotech Co., Ltd, China) to produce pET-*ipaH*, which was subsequently transformed into *E. coli* BL21(DE3). The cells were grown at 37°C to an OD₆₀₀ of 0.5 in LB supplemented with kanamycin (50 mg liter⁻¹). Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.1 mM. Cells were incubated for an additional 12 h at 16°C and then harvested by centrifugation and subjected to ultrasonic disruption (UH-650B ultrasonic processor; 40% intensity; Auto Science) for 10 min. Intact cells were removed by centrifugation at 12,000 × *g* for 30 min (4°C). A nickel-nitrilotriacetic acid (Ni²⁺-NTA) resin was used to purify the enzyme from the supernatant. A series of imidazole concentrations were used to elute the recombinant IpaH from the resin. SDS-PAGE was performed to determine the molecular weight of the protein, and the Bradford assay was used to quantify the protein concentration (40). Gel filtration chromatography was used to determine the native molecular mass of IpaH. Experiments were performed at a flow rate of 0.4 ml min⁻¹ using an AKTA purifier 10UPC system and a Superdex 200 10/300 GL column (GE Healthcare). The buffer used was 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl. The native molecular mass of IpaH was estimated from a calibration curve plotted by using the standard proteins, including thyroglobulin from porcine thyroid (669 kDa), ferritin from equine spleen (440 kDa), catalase from bovine liver (232 kDa), lactate dehydrogenase from bovine liver (140 kDa), bovine serum albumin (66 kDa), and cytochrome *c* (12.4 kDa).

Enzyme activity assay. The standard enzyme reaction was performed at 35°C for 10 min in 3 ml of 20 mM Tris-HCl buffer (pH 7.5) containing 0.6 mM iprodione and 0.006 μM IpaH. One unit of enzyme activity is defined as the amount of enzyme required to hydrolyze 1 μM iprodione per min.

Enzyme kinetics was studied using different concentrations of iprodione (2.27 to 63.60 μM) in the reaction mixture. The enzyme was diluted to 0.006 μM to ensure that the consumption of substrate was within the linear range during the reaction. The concentration of substrate was determined based on the integration of chromatographic peak areas observed during HPLC analysis. The *K_m* and *k_{cat}* values were calculated by nonlinear regression fitting to the Michaelis-Menten equation. All reactions were carried out in triplicate, and the data are reported as the means ± standard deviations.

Biochemical properties of the recombinant IpaH. The same concentration of IpaH and iprodione as that for the standard enzyme reaction was used to investigate the optimal temperature and pH of IpaH. To determine the optimal reaction temperature, IpaH activity was investigated at temperatures between 15°C and 70°C. The optimal reaction pH was assessed using several buffers with various pH values, including 20 mM disodium hydrogen phosphate-citric acid buffer (pH 3.0 to 7.0), 20 mM Tris-HCl (pH 7.0 to 9.0), and 20 mM glycine-NaOH buffer (pH 9.0 to 10.0). The thermal stability of IpaH was assessed by incubating the enzyme preparations at different temperatures for 1 h and then measuring the remaining activity under the enzyme assay conditions described above. Nonheated enzyme was used as the control (100%). To determine pH stability, IpaH was incubated at 4°C for 2 h in different pH buffers, and then the residual activity was measured. The samples were collected before iprodione was com-

pletely consumed. Activity observed by the standard enzyme was defined as 100%, and the relative activities of each reaction were calculated.

To identify whether IpaH is a metal-dependent enzyme, IpaH was treated with 0.1 mM, 1 mM, or 10 mM EDTA for 5 h at 4°C and then dialyzed against 20 mM Tris-HCl (pH 7.5) to remove the EDTA. Enzyme activity was assayed as described above and compared with the activity of IpaH without the treatment of EDTA.

The substrate specificity of IpaH was determined using indole-3-acetamide, propanil, 4-nitroacetanilide, leucine-*para*-nitroanilide, chlorpropham, carbendazim, linuron, benzamide, *N*-isopropylacetamide, *N*-acetyl glycine, L-glutamine, L-asparagine, acetamide, propanamide, and urea. The concentration of all the substrates was 0.6 mM, and the assay was conducted in standard enzyme reaction as outlined above.

Construction of *ipaH* gene-disrupted strain YJN-5. To disrupt *ipaH* through a single-crossover procedure (41), a 549-bp DNA fragment (in the middle of *ipaH*) was amplified from strain YJN-5 with primers KA-F and KA-R (Table 2). The fragment was cloned into the BamHI- and EcoRI-digested pEX18Gm plasmid (42) using the ClonExpressII one-step cloning kit to produce pEX-*ipaH*. The corresponding plasmid was introduced into the strain YJN-5 by electroporation, as described by Zhang et al. (43). Single-crossover clones were selected on LB plates supplemented with streptomycin (50 mg liter⁻¹) and gentamicin (50 mg liter⁻¹). The *ipaH* insertion mutant, designated YJN-5M, was verified by PCR with primers KT-F and KT-R (Table 2), and the resulting amplicons were sequenced to confirm the successful integration of pEX-*ipaH* in strain YJN-5M. Its ability to degrade iprodione was tested in MSM supplemented with 0.6 mM iprodione.

Complementation of the *ipaH*-disrupted mutant. A 1,701-bp fragment containing the 291-bp region just upstream of *ipaH* was amplified from strain YJN-5 using primers CP-F and CP-R (Table 2). The PCR product was cloned into the BamHI- and EcoRI-digested broad-host-range plasmid pBBR1MCS-2 (44) using the ClonExpressII one-step cloning kit to produce pBBR1-*ipaH*, which was then introduced into the mutant strain YJN-5M through electroporation to generate YJN-5M(pBBR1-*ipaH*). The strain's ability to degrade iprodione was tested in MSM supplemented with 0.6 mM iprodione.

Site-directed mutagenesis. Point mutations in *ipaH* were constructed by overlap PCR. Primers IH-F and IH-R were used as the forward and reverse flanking primers, respectively. The internal primer pairs K82A-F/R, S157A-F/R, and S181A-F/R are shown in Table 2. All PCR assays were performed with the Phanta Max super-fidelity DNA polymerase (Vazyme Biotech Co., Ltd, China) and a standard site-directed mutagenesis protocol (45). The PCR products were gel purified and then subsequently cloned into the NdeI and XhoI sites of the pET-29a(+) plasmid, as described above. Successful substitutions were confirmed by DNA sequencing. Purification of the recombinant proteins and analysis of their activity against iprodione were performed as previously described.

Analytical methods. To analyze iprodione and its metabolites, the culture or enzyme assay samples were extracted with equal volumes of acetonitrile and then centrifuged at 12,000 × *g* for 5 min. The supernatants were filtered through a 0.2-μm-pore-size filter. Iprodione concentrations were determined using a high-performance liquid chromatography (HPLC) system (Dionex UltiMate 3000, USA) equipped with a C₁₈ reverse-phase column (4.6 by 250 nm, 5 μm). The mobile phase consisted of acetonitrile-water-acetic acid (70:30:0.5, vol/vol/vol) at a flow rate of 0.8 ml min⁻¹. Column elution was monitored by measuring the absorbance at 235 nm. The injection volume was 20 μl. The column temperature was maintained at 30°C. For identification of the intermediate metabolites, the mass spectrum was collected using a TripleTOF 5600 (AB SCIEX) mass spectrometer. The metabolites were ionized by electrospray with positive polarity, and characteristic fragment ions were detected using MS/MS. The mass spectra of the authentic *N*-(3,5-dichlorophenyl)-2,4-dioximidazolidine (compound II) and 3,5-dichloroaniline (compound IV) were analyzed under the same conditions, and the results were used as the standards (see Fig. S14 in the supplemental material). As the authentic (3,5-dichlorophenyl)urea acetic acid (compound III) was not available, the analysis of its MS/MS fragments was also shown in Fig. S14.

To investigate the substrate spectrum of IpaH, the amidase activity of IpaH against substrates (indole-3-acetamide, benzamide, L-glutamine, L-asparagine, acetamide, propanamide, and urea) was determined by the release of ammonia using the phenol-hypochlorite ammonia detection method (46). Propanil, 4-nitroacetanilide, leucine-*para*-nitroanilide, chlorpropham, carbendazim, and linuron were detected by HPLC, and the mobile phase consisted of methanol-water (80:20, vol/vol) at a flow rate of 0.8 ml min⁻¹. Column elution was monitored by measuring the absorbance at 230 nm. *N*-Isopropylacetamide and *N*-acetyl glycine were also detected by HPLC, with a mobile phase consisting of acetonitrile-water (50:50, vol/vol) and a flow rate of 0.8 ml min⁻¹. Column elution was monitored by measuring the absorbance at 215 nm.

Accession number(s). The 16S rRNA gene sequence and the DNA fragment (10,850 bp) containing the amidase gene *ipaH* from strain YJN-5 were deposited in the GenBank database under accession numbers MG733131 and MG601458, respectively. The GenBank accession number for the amidase gene *ipaH* of strain YJN-G is MG601459.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01150-18>.

SUPPLEMENTAL FILE 1, PDF file, 2.6 MB.

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