Detoxification Esterase StrH Initiates Strobilurin Fungicide Degradation in *Hyphomicrobium* sp. Strain DY-1

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ABSTRACT Strobilurin fungicides are widely used in agricultural production due to their broad-spectrum and fungal mitochondrial inhibitory activities. However, their massive application has restrained the growth of eukaryotic algae and increased collateral damage in freshwater systems, notably harmful cyanobacterial blooms (HCBs). In this study, a strobilurin fungicide-degrading strain, *Hyphomicrobium* sp. strain DY-1, was isolated and characterized successfully. Moreover, a novel esterase gene, *strH*, responsible for the de-esterification of strobilurin fungicides, was cloned, and the enzymatic properties of StrH were studied. For trifloxystrobin, StrH displayed maximum activity at 50°C and pH 7.0. The catalytic efficiencies (k_{cat}/K_m) of StrH for different strobilurin fungicides were $196.32 \pm 2.30 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ (trifloxystrobin), $4.64 \pm 0.05 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ (picoxystrobin), $2.94 \pm 0.02 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ (pyraclostrobin), and $(2.41 \pm 0.19) \times 10^{-2} \mu\text{M}^{-1} \cdot \text{s}^{-1}$ (azoxystrobin). StrH catalyzed the de-esterification of a variety of strobilurin fungicides, generating the corresponding parent acid to achieve the detoxification of strobilurin fungicides and relieve strobilurin fungicide growth inhibition of *Chlorella*. This research will provide insight into the microbial remediation of strobilurin fungicide-contaminated environments.

IMPORTANCE Strobilurin fungicides have been widely acknowledged as an essential group of pesticides worldwide. So far, their residues and toxic effects on aquatic organisms have been reported in different parts of the world. Microbial degradation can eliminate xenobiotics from the environment. Therefore, the degradation of strobilurin fungicides by microorganisms has also been reported. However, little is known about the involvement of enzymes or genes in strobilurin fungicide degradation. In this study, a novel esterase gene responsible for the detoxification of strobilurin fungicides, *strH*, was cloned in the newly isolated strain *Hyphomicrobium* sp. DY-1. This degradation process detoxifies the strobilurin fungicides and relieves their growth inhibition of *Chlorella*.

KEYWORDS strobilurin fungicides, *Hyphomicrobium* sp. DY-1, detoxification esterase, biodegradation

Strobilurin fungicides, an essential group of pesticides, have been widely acknowledged for controlling fungal pathogens in crops. They have an estimated annual sale of \$3.4 billion and account for 25% of the fungicides sold worldwide (1). The mode of action in fungi involves binding to the quinol oxidation site of cytochrome *b* in the mitochondrial electron transfer chain, which leads to the inhibition of metabolic energy (ATP) production, followed by the death of the fungi (2). The representative species of strobilurin fungicides include azoxystrobin, pyraclostrobin, trifloxystrobin, and picoxystrobin (see Fig. S1 in the supplemental material). The residues of strobilurin fungicides can be detected in different parts of the world due to their extensive usage. Azoxystrobin has been detected in freshwater ecosystems (within a 0.01- to 29.70- $\mu\text{g} \cdot \text{liter}^{-1}$ concentration) in streams, ponds, groundwater, and lakes in Denmark, Germany,

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France, Brazil, and the United States (3–5). Similarly, pyraclostrobin has been detected at a concentration of $17.24 \mu\text{g} \cdot \text{liter}^{-1}$ in China's paddy water (6).

Strobilurin fungicides have toxic effects on several aquatic organisms (3, 7, 8), such as *Daphnia magna*. These fungicides are able to harm *D. magna* at environmentally relevant concentrations (0.15 to $20 \mu\text{g} \cdot \text{liter}^{-1}$) (9). Compared with cyanobacteria, eukaryotic algae are particularly sensitive to strobilurin fungicides (10). Trifloxystrobin affects the antioxidant activities, cellular structure, and photosynthesis of *Chlorella vulgaris* (11, 12). Lu et al. found that azoxystrobin and pyraclostrobin contamination inhibits the growth of cyanobacterial competitors' green algae and potentially contributes to cyanobacterial dominance and harmful cyanobacterial blooms (HCBs) (13, 14). HCBs lead to bottom-water oxygen depletion and the accumulation of cyanotoxins (microcystins, antitoxins, etc.), which have a negative effect on diverse ecosystems, including habitat loss, economic costs (fisheries, recreation, and tourism losses), and human health risks (15–17). In this regard, the environmental behavior and degradation mechanism of strobilurin fungicides have attracted great concern and interest.

Microbial degradation plays a vital role in the dissipation of strobilurin fungicides. To date, several strobilurin fungicide-degrading microorganisms have been isolated. Lopes et al. isolated a *Klebsiella* strain capable of degrading pyraclostrobin (18). Clinton et al. isolated several strains, including *Stenotrophomonas maltophilia*, *Bacillus amyloliquefaciens*, *Bacillus flexus*, and *Arthrobacter oxydans*, which were able to utilize trifloxystrobin as a carbon source (19). Howell et al. isolated two bacterial strains, *Rhodanobacter* sp. strain CCH1 and *Cupriavidus* sp. strain CCH2, which were able to degrade azoxystrobin when it was supplied as the sole carbon source (20). Additionally, Feng et al. isolated a bacterial strain, *Ochrobactrum anthropi* SH14, which was able to utilize azoxystrobin as the sole carbon source and degrade a wide range of strobilurin fungicides (21). Although there are several reports on the identification of metabolites that appeared during the degradation of strobilurin fungicide (21–23), no degradation pathway, enzymes, or genes have been reported for strobilurin fungicides (Fig. S2). In this study, a novel esterase gene, *strH*, was cloned in the newly isolated strobilurin fungicide-degrading strain DY-1, and the characteristics of StrH were studied. StrH catalyzed the de-esterification of strobilurin fungicides, generating the corresponding parent acids. This degradation process detoxifies the strobilurin fungicides and relieves their growth inhibition of *Chlorella*. This study will provide strain resources and a theoretical basis for controlling strobilurin fungicide pollution in freshwater through microbial remediation.

RESULTS

Isolation and identification of a trifloxystrobin-degrading strain. A trifloxystrobin-degrading bacterium, strain DY-1, was isolated from the soil through enrichment cultures. This strain formed a clear transparent halo on R2A plates amended with 0.15 mM trifloxystrobin (see Fig. S3 in the supplemental material). Its colonies on R2A plates were smooth, creamy, oval, and Gram stain negative, with dimensions of 0.6 to 0.7 by 1.3 to $2.4 \mu\text{m}$ (Fig. S3). Strain DY-1 was positive for the following activities or reactions: urease and utilization of adonitol, D-maltose, D-mannitol, Palatinose, saccharose, D-tagatose, and D-trehalose as carbon sources. It was negative for Ala-Phe-Pro arylamidase, L-pyrrolidonyl arylamidase, glutamyl arylamidase pNA, and the utilization of L-arabinose, D-cellobiose, D-glucose, D-mannose, and D-sorbitol. These characteristics were consistent with the general properties of *Hyphomicrobium* species (24). The 16S rRNA gene sequence of DY-1 was highly similar to those of the known *Hyphomicrobium* strains, including *Hyphomicrobium facile* subsp. *facile* ATCC 27485^T (99.14%), *Hyphomicrobium facile* subsp. *ureaphilum* ATCC 27492^T (99.07%), *Hyphomicrobium facile* subsp. *tolerans* ATCC 27489^T (99.07%), *Hyphomicrobium denitrificans* ATCC 51888^T (98.43%), *Hyphomicrobium methylovorum* (98.22%), and *Hyphomicrobium chloromethanicum* CM2^T (98.15%). A phylogenetic tree constructed based on the 16S rRNA gene sequences of strain DY-1 and its close relatives is

FIG 1 Growth of strain DY-1 in MSM, MSM with methanol (0.15 mM), and MSM with trifloxystrobin (0.15 mM). The initial inoculum of strain DY-1 was 1.15×10^{-7} CFU $\cdot$ ml⁻¹. Error bars represent the standard errors from three replicates.

presented in Fig. S4. Based on the above information, strain DY-1 was preliminarily identified as a *Hyphomicrobium* sp.

Degradation of strobilurin fungicides by strain DY-1. Strain DY-1 was able to degrade 0.15 mM trifloxystrobin in 20 h (Fig. S5), and weak growth was observed for strain DY-1 during the degradation process (Fig. 1). The optimal activity to degrade trifloxystrobin was observed at pH 8.0 and 35°C (Fig. S6). Besides, strain DY-1 could also degrade other strobilurin fungicides, including azoxystrobin, pyraclostrobin, and picoxystrobin, but it degrades trifloxystrobin the fastest and azoxystrobin the slowest (Fig. S5).

Analysis and identification of trifloxystrobin metabolites. Of the samples collected 12 h after the inoculation of strain DY-1, only two compounds (compounds I and II), with retention times of 5.30 min and 8.71 min, were detected through high-performance liquid chromatography (HPLC). With the progress of the degradation process, the peak height of compound I continued to decrease until it completely disappeared at 20 h. Similarly, the peak height of compound II continued to increase until it reached a constant height, which remained unchanged afterward, irrespective of the extended time, suggesting it to be the end product (Fig. 2A). Tandem mass spectrometry (MS/MS) analysis was employed to identify the two above-mentioned compounds (I and II). The prominent protonated molecular ion of compound I was 409.1367 [M+H]⁺ and identified as trifloxystrobin (C₂₀H₂₀F₃N₂O₄⁺, *m/z* 409.1370), with a 0.7-ppm error (Fig. 2B). The molecular ion peak [M+H]⁺ of compound II was observed at *m/z* 395.1209, which was consistent with the protonated derivative of trifloxystrobin acid (C₁₉H₁₈F₃N₂O₄⁺, *m/z* 395.1213), with a 1.0-ppm error (Fig. 2C). In general, a mass error between -5 and +5 ppm is acceptable for the identification of compounds (25). Also, the mass spectrometric identification result of authentic trifloxystrobin and trifloxystrobin acid under the same conditions proves the conclusion (Fig. S7). Therefore, we proposed a putative degradation pathway of trifloxystrobin in strain DY-1 (Fig. 2D) in which trifloxystrobin was hydrolyzed to trifloxystrobin acid and methanol. Although methanol is not detected, strain DY-1 was able to use methanol as the sole carbon source for growth. However, strain DY-1 showed weak growth in mineral salts medium (MSM) with 0.15 mM methanol as the sole carbon source. This result was consistent with the results of MSM with 0.15 mM trifloxystrobin (Fig. 1). Moreover, when the methanol concentration was increased to 0.5 M, strain DY-1 showed an obvious growth (Fig. S8).

Cloning and sequence analysis of the *strH* gene. A positive clone designated *Escherichia coli* pE producing a transparent halo around the colony was screened from approximately 10,000 transformants in the genomic library (Fig. S9A). Further, its ability to degrade trifloxystrobin was further confirmed by HPLC-MS. The result showed that strain *E. coli* pE could transform trifloxystrobin to trifloxystrobin acid (Fig. S9BCD). This was consistent with the hydrolysis step of trifloxystrobin in strain DY-1. The

FIG 2 Degradation pathway of trifloxystrobin in strain DY-1. (A) HPLC analysis of metabolites that appeared during the degradation of trifloxystrobin by strain DY-1; (B) MS/MS analysis of compound I (m/z 409.1367 $[M+H]^+$), which was identified as trifloxystrobin; (C) MS/MS analysis of compound II (m/z 395.1209 $[M+H]^+$), which was identified as trifloxystrobin acid; (D) metabolic pathway of trifloxystrobin in strain DY-1. mAU, arbitrary units (in thousands).

recombinant plasmid harbored by *E. coli* pE was named pE. Its inserted fragment was 3,247 bp and contained two complete open reading frames (ORFs), designated *strH* and *orf1*. The physical map of this fragment is depicted in Fig. S10. The protein sequences encoded by these ORFs were used as queries in a BLASTP search (UniProtKB/Swiss-

TABLE 1 Kinetic parameters of recombinant StrH

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1} \text{s}^{-1}$)
Trifloxystrobin	4.47 ± 0.47	877.55 ± 10.31	196.32 ± 2.30
Picoxystrobin	12.35 ± 0.70	57.32 ± 0.64	4.64 ± 0.05
Azoxystrobin	30.26 ± 4.85	0.73 ± 0.06	$(2.41 \pm 0.19) \times 10^{-2}$
Pyraclostrobin	16.37 ± 3.84	48.14 ± 0.32	2.94 ± 0.02

Prot database), and the functions were proposed for each ORF. *orf1* encoded a protein consisting of 332 amino acid residues sharing 28% similarity with the transcriptional activator FeaR (NCBI Protein accession no. [Q47129](#)) from strain *Escherichia coli* K-12 (26). The *strH* gene is 1,713 bp long and encodes a protein consisting of 570 amino acids. The deduced StrH protein shared a low amino acid sequence identity (28% to 35%) with several biochemically characterized enzymes obtained from *Sphingopyxis macrogoltabida* (fumonis B1 esterase, accession no. [D2D3B6](#), 35% identity) (27), *Thermobifida fusca* (carboxylesterase, accession no. [Q47M62](#), 28% identity) (28), *Arthrobacter oxydans* (phenmedipham hydrolase, accession no. [Q01470](#), 30% identity) (29), and *Bacillus subtilis* (*p*-nitrobenzyl esterase, accession no. [P37967](#), 35% identity) (30). The phylogenetic analysis based on the amino acid sequences of StrH and its closely related proteins demonstrated that StrH belongs to the esterase VII family and forms a clade with other esterases in this family, thereby suggesting a close evolutionary relationship (Fig. S11) (31). Additionally, StrH hydrolyzed 4-nitrophenyl acetate, causing a change in color from colorless to yellow (Fig. S12). These results also confirmed that StrH was a member of the esterase family.

Heterogeneous expression of *strH*. The *strH* gene was cloned and expressed in *E. coli* BL21(DE3) to investigate whether StrH is responsible for hydrolyzing the ester bond of trifloxystrobin in strain DY-1. Recombinant StrH was purified from the crude extract using streptavidin-berpharose FF, which appeared as a single band by SDS-PAGE (Fig. S13). The molecular mass of the denatured enzyme was approximately 61 kDa, which was consistent with the theoretical molecular mass (61 kDa). StrH catalyzed the de-esterification of a variety of strobilurin fungicides (trifloxystrobin, azoxystrobin, pyraclostrobin, picoxystrobin), generating the corresponding parent acid (Fig. S14, S15, S16, and S17). Therefore, it was confirmed that StrH is responsible for the hydrolysis of the ester bond of strobilurin fungicides.

Biochemical characterization of StrH. The optimal activity of StrH was observed at pH 7.0 and 50°C. The enzyme was stable up to 40°C, had 20% residual activity at 50°C, and then was completely inactivated at 60°C for 1 h. The enzyme was stable at pH 5.0 to 7.0, retaining more than 90% of the original activity after preincubation at the same pH range for 1 h (Fig. S18). The activity of StrH was not affected by EDTA in the tested concentrations, indicating that the enzyme is not a metalloesterase. StrH was capable of hydrolyzing all the tested strobilurin fungicides, with the hydrolysis rates (k_{cat}/K_m) descending as follows: trifloxystrobin > picoxystrobin > pyraclostrobin > azoxystrobin (Table 1).

Transcriptional level of *strH* in strain DY-1 under the induction of trifloxystrobin. The relative changes in *strH* transcription in strain DY-1 under trifloxystrobin-induced and uninduced conditions were investigated by quantitative real-time PCR. The data elucidated that there was no significant difference between the cells grown under induced and uninduced conditions (Fig. S19). It confirmed that the expression of *strH* in strain DY-1 was conservative.

Conserved amino acid sites of StrH. Alignment of the amino acid sequences of StrH and its closely related proteins (identity, 30% to 34%) with available crystal structures in the Protein Data Bank (PDB) revealed the conserved amino acid sites (Fig. S20). Of these, catalytic triplet S-E-H (Ser232, Glu354, His467) in StrH (represented by the green pentagon) was replaced by alanine using the overlap PCR method. The three mutants obtained were expressed heterologously in *E. coli* BL21(DE3) and purified,

FIG 3 Detoxification of strobilurin fungicides by strain DY-1. The initial cell densities for *C. ellipsoidea* and strain DY-1 were set at 0.01 (OD₆₈₀) and 1.15×10^7 CFU · ml⁻¹, respectively. The strobilurin fungicides (trifloxystrobin, picoxystrobin, pyraclostrobin, and azoxystrobin) were added at a concentration of 1 mg · liter⁻¹. An asterisk indicates that there is a significant difference compared with the strobilurin fungicide treatment as determined by the unpaired *t* test (*, *P* < 0.05). A red asterisk indicates that there is a significant difference between the value of the point on the black line or the blue line and that on the red line. A blue asterisk indicates that there is a significant difference between the value of the point on the black line and that on the blue line.

resulting in three variants (StrH-S232A, StrH-E354A, and StrH-H467A) (Fig. S21). The enzyme activity assay revealed that all the variants lost hydrolysis activity against trifloxystrobin. These results further prove that StrH is an esterase enzyme containing the highly conserved catalytic triad Ser-Glu-His.

Detoxification assay of strobilurin fungicides by strain DY-1. Three treatments, including treatment 1 (T1) (*Chlorella ellipsoidea* plus 1 mg · liter⁻¹ different strobilurin fungicides), T2 (*C. ellipsoidea* plus 1 mg · liter⁻¹ different strobilurin fungicides plus strain DY-1), and T3 (*C. ellipsoidea* plus strain DY-1), were employed to investigate the detoxification effect of strobilurin fungicides on strain DY-1. For trifloxystrobin treatment, the growth of *C. ellipsoidea* in T1 was completely inhibited until the 12th day. However, there was no difference between the levels of growth of *C. ellipsoidea* in T2 and T3, indicating a strong inhibitory effect of trifloxystrobin on the growth of *C. ellipsoidea*, while the addition of strain DY-1 completely relieved the inhibitory effect (Fig. 3A). For picoxystrobin treatment, the growth of *C. ellipsoidea* in T1 was also completely inhibited until the 12th day. The growth of *C. ellipsoidea* in T2 was better than that in T1 but had a certain lag period compared to that in T3. This observation suggested a strong inhibitory effect of picoxystrobin on the growth of *C. ellipsoidea*, while the addition of strain DY-1 partially relieved the inhibitory effect (Fig. 3B). For pyraclostrobin treatment, the growth of *C. ellipsoidea* in T1 showed a lag period compared to that in T2, but there was no difference between levels of growth in T2 and T3. These results revealed that pyraclostrobin had a moderate inhibitory effect on the growth of *C. ellipsoidea*, while the addition of strain DY-1 completely relieved the inhibitory effect and restored growth (Fig. 3C). For azoxystrobin treatment, the growth of *C. ellipsoidea* in T1 showed a lag period compared to that in T2, but there was no difference between levels of growth in T1 and T3. These results suggested that azoxystrobin had a moderate inhibitory effect on the growth of *C. ellipsoidea*, but the original addition quantity of strain DY-1 (1.15×10^7 CFU · ml⁻¹) could not relieve the effect at all (Fig. 3D). However,

the increased inoculation amount of strain DY-1 (1.15×10^8 CFU $\cdot$ ml $^{-1}$) relieved the inhibitory effect of azoxystrobin (Fig. S22). All these results demonstrated that the tested strobilurin fungicides were able to inhibit the growth of *Chlorella ellipsoidea* but that the degrees of inhibition were different. The addition of strain DY-1 relieved the inhibition of *C. ellipsoidea*, while its alleviation effects differed.

DISCUSSION

To date, several strobilurin fungicide-degrading bacteria have been reported from the genus *Klebsiella*, *Bacillus*, *Arthrobacter*, *Stenotrophomonas*, *Rhodanobacter*, *Cupriavidus*, and *Ochrobactrum* (18–21). Of them, the degradation characteristics have been studied for only a few strains. For instance, *Klebsiella* sp. strain 1805 was able to completely transform 36.5 μ M pyraclostrobin in 120 h. *Rhodanobacter* sp. strain CCH1 and *Cupriavidus* sp. strain CCH2 were able to degrade 12 μ M trifloxystrobin within 20 h, as well as pyraclostrobin and azoxystrobin (20). *Ochrobactrum anthropi* strain SH14 degraded 86.3% of azoxystrobin (0.12 mM) in an MSM within 5 days and other strobilurin fungicides, including kresoxim-methyl, pyraclostrobin, trifloxystrobin, and picoxystrobin (21). In this study, strain DY-1 was isolated from the genus *Hyphomicrobium*, enriching the diversity of strobilurin fungicide-degrading strains. Strain DY-1 was capable of degrading 0.15 mM trifloxystrobin, pyraclostrobin, picoxystrobin, and azoxystrobin within 20 h, 83 h, 96 h, and 312 h, respectively (see Fig. S5 in the supplemental material). It degraded strobilurin fungicides through the hydrolysis step, resulting in the product, i.e., the parent acid and methanol (Fig. 2). This degradation step was consistent with the hydrolysis of trifloxystrobin in the environment described by Banerjee et al. (22). This result indicates that the residual strobilurin fungicides in the environment were able to be degraded by microorganisms or photolysis to produce the corresponding parent acid.

Hyphomicrobium strains are methylotrophs that can grow with methanol (32, 33). They are ubiquitous in water and soil and also exist in sewage treatment plants. In recent years, several strains of the genus *Hyphomicrobium*, especially those capable of degrading environmental pollutants, such as *Hyphomicrobium* sp. strain MAP-1 (capable of degrading methamidophos) and *Hyphomicrobium chloromethanicum* CM2 (able to degrade chloromethane and utilize it as the sole carbon source for growth) have been reported (34, 35). In this study, strobilurin fungicides were degraded by strain *Hyphomicrobium* sp. DY-1, and weak growth of strain DY-1 was observed during the degradation process (Fig. 1). The possible reason of weak growth is that the quantity of methanol produced during the hydrolysis of strobilurin fungicides (0.15 mM) in the cell maintained only the basic metabolism. Although strain DY-1 was able to utilize methanol for its growth, the growth was still weak at a concentration of 0.15 mM (consistent with the result of 0.15 mM trifloxystrobin). Strain DY-1 was capable of utilizing methanol (0.5 M) as the sole carbon source for significant growth (Fig. S8). The water solubility of strobilurin fungicides is low (0.6 to 6 mg $\cdot$ liter), so it is difficult to increase the concentration of strobilurin fungicides to achieve the significant growth of strain DY-1.

Esterase plays a significant role in the hydrolysis of a wide range of ester-containing xenobiotics. The esterase genes involved in the hydrolysis of several pesticides and herbicides, such as phenmedipham, pyrethroids, parathion, carbaryl, carbendazim, carbofuran, and phenylurea, have been cloned and characterized (29, 36–41). In this study, StrH was proved to have the ability to hydrolyze strobilurin fungicides. StrH belongs to the esterase VII family protein (Fig. S11) and displays the highest conserved esterase domain, G-X-S-X-G, and the catalytic triad S-E-H in StrH (Fig. S20) (31). However, StrH shows only moderate amino acid sequence homology with other characterized esterase VII family proteins (Fig. S11). These results suggest that StrH differs from the previously reported esterases by the substrate difference. Interestingly, the amino acid sequence alignment analysis of StrH in the nonredundant (NR) protein sequence database in the NCBI showed that StrH has homologous proteins (68% to 72% similarity) in the sequenced genomes of the strains of the genus *Hyphomicrobium*.

Although the functions of these proteins have not been identified, this result suggests that other strains in the *Hyphomicrobium* genus might also have the ability to degrade strobilurin fungicides.

StrH was able to de-esterify a wide range of strobilurin fungicides to produce corresponding parent acids (Fig. S14 to S17), but their catalytic efficiencies (k_{cat}/K_m) differ from each other. StrH exhibited the best catalytic activity for trifloxystrobin, moderate activity for picoxystrobin and pyraclostrobin, and the lowest activity for azoxystrobin, which was a thousand times lower than that for trifloxystrobin. The strobilurin fungicides were similar in their ester bond structures but differed in their side chain groups. This might have happened because of their different side chain structures, which led to the differences in the catalytic efficiencies of StrH against them. Some other esterases also showed similar characteristics (42, 43). The sulfonylurea herbicide de-esterification esterase SulE could hydrolyze a variety of sulfonylurea herbicides with a methyl or ethyl ester as the substrate of the enzyme with different hydrolysis rates (42). The enzyme catalytic efficiency of SulE against thifensulfuron-methyl was $974.4 \text{ mM}^{-1} \cdot \text{s}^{-1}$, but it was $51.9 \text{ mM}^{-1} \cdot \text{s}^{-1}$ against chlorimuron-ethyl (42). The pyrethroid-hydrolyzing carboxylesterase PytH could hydrolyze various pyrethroids as substrates with different catalytic efficiencies (36). Also, the carbamate insecticide hydrolase CehA could hydrolyze a variety of carbamate insecticides with different catalytic efficiencies (43).

Given that strobilurin fungicides will continue to be widely used, how to eliminate the harm of their residues is a scientific issue worthy of attention (9, 12–14). Previous studies have reported that strobilurin fungicides were able to affect the growth of eukaryotic algae, the ecological balance of water bodies, and the frequency of HCBs (13, 14). Strain DY-1 was able to detoxify a variety of strobilurin fungicides, including trifloxystrobin, picoxystrobin, pyraclostrobin, and azoxystrobin and relieved their growth inhibition of eukaryotic *Chlorella* organisms in an aquatic model (Fig. 3). However, the detoxification effect was different. Strain DY-1 showed the best detoxification effect on trifloxystrobin and pyraclostrobin and restored the growth of *C. ellipsoidea* (Fig. 3A to C). However, a moderate detoxification effect on picoxystrobin was able to partially restore the growth of *C. ellipsoidea* (Fig. 3B). The same inoculum amount of strain DY-1 ($1.15 \times 10^7 \text{ CFU} \cdot \text{ml}^{-1}$) showed no significant detoxification effect on azoxystrobin (Fig. 3D). It is due to the relatively poor degradation efficiency on azoxystrobin by strain DY-1, which took more than 300 h to degrade 0.15 mM strobilurin (Fig. S5D) and 20 h to degrade the same concentration of trifloxystrobin (Fig. S5A). Nevertheless, the good detoxification effect of azoxystrobin could be achieved by increasing the inoculation amount of strain DY-1 up to $1.15 \times 10^8 \text{ CFU} \cdot \text{ml}^{-1}$ (Fig. S22). Collectively, this study isolated a strobilurin fungicide-degrading strain, DY-1, which could detoxify them through de-esterification by StrH. This process was able to relieve the inhibition by strobilurin fungicides of the growth of *C. ellipsoidea* and provide a theoretical basis for the removal of strobilurin fungicide pollution in freshwater.

MATERIALS AND METHODS

Chemicals and media. Trifloxystrobin (purity 98%), azoxystrobin (purity 98.5%), picoxystrobin (purity 98%), pyraclostrobin (purity 98%), and trifloxystrobin acid (98%) were purchased from J&K Scientific Ltd. (Shanghai, China). R2A (Sigma-Aldrich 17209) and BG-11 (Sigma-Aldrich C3061) media were purchased from Sigma-Aldrich Co. (Shanghai, China). Lysogeny broth (LB) consisted of the following components (in grams per liter): tryptone (10.0), yeast extract (5.0) and NaCl (10.0), pH 7.0. Mineral salts medium (MSM) consisted of the following components: $0.5 \text{ g} \cdot \text{liter}^{-1} \text{ K}_2\text{HPO}_4$, $1.5 \text{ g} \cdot \text{liter}^{-1} \text{ KH}_2\text{PO}_4$, $1.0 \text{ g} \cdot \text{liter}^{-1} \text{ NaCl}$, $1.0 \text{ g} \cdot \text{liter}^{-1} \text{ NH}_4\text{Cl}$, and $0.2 \text{ g} \cdot \text{liter}^{-1} \text{ MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 7.0. The strobilurin fungicide stock solutions ($10,000 \text{ mg} \cdot \text{liter}^{-1}$) were prepared in acetone and sterilized by membrane filtration with a filter pore size of $0.22 \mu\text{m}$, as they are incapable of utilizing acetone as a carbon source for growth. All other chemical reagents were of the highest analytical purity.

Strains, plasmids, and culture conditions. The strains and plasmids used in this study are listed in Table 2, and the primers are listed in Table 3. *Escherichia coli* strains were grown on LB on a rotary shaker (180 rpm) or on LB agar (1.5%, wt/vol) plates at 37°C . Strain DY-1, deposited in the China Center for Type Culture Collection (deposition no. CCTCC M 2020190), was cultivated aerobically in R2A broth at 30°C . Strains of the green alga *Chlorella ellipsoidea* (FACHB-40), obtained from the Institute of Hydrobiology at the Chinese Academy of Sciences (Wuhan, China), were cultivated on BG-11 medium. The microcosms

TABLE 2 Strains and plasmids used in this study

Strain or plasmid	Characteristic(s) ^a	Source
Strains		
<i>Hyphomicrobium</i> sp. DY-1	Degrades trifloxystrobin	This study
<i>Chlorella ellipsoidea</i> (FACHB-40)		Institute of Hydrobiology at the Chinese Academy of Sciences
<i>E. coli</i> DH5 α	F ⁻ <i>recA1 endA1 thi-1 supE44 relA1 deoR</i> Δ (<i>lacZYA-argF</i>) <i>U169</i> ϕ 80 Δ <i>lacZ</i> Δ M15	Vazyme
<i>E. coli</i> BL21	F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻) dcm gal</i> λ (DE3)	Vazyme
Plasmids		
pMD19-T	TA clone vector, Amp ^r	TaKaRa
pUC118 BamHI/BAP	DNA library construction vector, Amp ^r	TaKaRa
pET-29a(+)	Expression vector, Km ^r	This study
pET- <i>strH</i>	pET-29a(+) derivative carrying <i>strH</i> , Km ^r	This study
pET- <i>strH</i> -S232A	pET-29a(+) derivative carrying <i>strH</i> -S232A, Km ^r	This study
pET- <i>strH</i> -E354A	pET-29a(+) derivative carrying <i>strH</i> -E354A, Km ^r	This study
pET- <i>strH</i> -H467A	pET-29a(+) derivative carrying <i>strH</i> -H467A, Km ^r	This study

^aAmp^r, ampicillin resistant; Km^r, kanamycin resistant.

were placed in an artificial greenhouse at 25 $\pm$ 0.5°C under cool-white fluorescent light (46 μ mol $\cdot$ photons m⁻² $\cdot$ s⁻¹), with a 12-h/12-h light/dark cycle.

Isolation and identification of a trifloxystrobin-degrading strain. A trifloxystrobin-degrading strain was isolated by the enrichment culture technique. Soil samples were collected from trifloxystrobin-applied farmland in Anhui Province, China. Approximate 5.0 g of soil sample was placed in a 250-ml Erlenmeyer flask containing 100 ml of MSM supplemented with 0.15 mM trifloxystrobin as the carbon source. The culture was incubated at 30°C on a rotary shaker at 180 rpm for approximately 4 days. Five milliliters of the enrichment culture was then transferred to fresh MSM supplemented with 0.15 mM trifloxystrobin every 4 days for subculture. After 5 rounds of enrichment, three colonies capable of producing transparent hydrolysis halos were obtained by spreading serially diluted enrichment cultures on R2A plates containing 0.15 mM trifloxystrobin. However, the 16S rRNA gene sequence analysis, comparison of colony morphologies, and degradation efficiency analysis showed no significant differences between them. Therefore, we speculated that these three colonies originated from the same strain, i.e., DY-1.

The identification of strain DY-1 was conducted according to protocols in *Bergey's Manual of Determinative Bacteriology* (44) and sequence analysis of the 16S rRNA gene. Cell morphology and dimensions were determined by light microscopy (model CX23; Olympus) and transmission electron microscopy (model H-7650; Hitachi) (45). Utilization of various substrates, enzyme activities, and other physiological and biochemical properties were tested using the Vitek 2 Gram-negative identification card. For 16S rRNA gene sequencing and phylogenetic analysis, the genomic DNA was extracted using a bacterial genomic DNA mini-kit (Sangon, Shanghai, China). The universal bacterial primers 27F and 1492R were used for amplification of the 16S rRNA gene, and the purified PCR product was cloned into the pMD19-T vector (TaKaRa, Beijing, China) (45). Later, it was transformed into competent *Escherichia coli* DH5 α cells and sequenced by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). The 16S rRNA gene sequence of strain DY-1 was compared

TABLE 3 Primers used in this study

Primer	DNA sequence (5' to 3')	Description
27F	AGAGTTTGATCCTGGCTCAG	Amplification of 16S rRNA gene
1492R	GGTCCCTTGTACGACTT	
StrH-F	AAGAAGGAGATATACATATGCAAAGTATCTTCAATCTC	Construction of plasmid pET- <i>strH</i>
StrH-R	GGTGGTGGTGGTCTCGAGTCACCTTTTCGAAGTGC GG GTGGCTC CAGTAGCCTGCAACCGATC	
RT16S-F	AGGTGGATTGTGAAGTCAG	Real-time PCR to amplify a fragment of the 16S rRNA gene
RT16S-R	ACATGCTCCACCGCTTGTG	
RTSH-F	TGCGCGCTGCATACTGATC	Real-time PCR to amplify a fragment of <i>strH</i>
RTSH-R	TTCGACACAGCCATTCCAG	
S232A-F	CAAGCTGCAGGCGCGCTC	Amplification of <i>strH</i> -S232A
S232A-R	GAGCGCGCTGCAGCTTG	
E354A-F	GACCAAAGACGCGGGCAC	Amplification of <i>strH</i> -E354A
	GTGCCCCGCTCTTTGGTC	
H467A-F	CTGGCAGCAGCCACGATTG	Amplification of <i>strH</i> -H467A
H467A-R	CAATCGTGGCTGCTGCCAG	

with those available from the EzTaxon database (46). The phylogenetic analysis was performed using the software package MEGA version 7.0 (47) after multiple alignments of data through CLUSTAL_X. The phylogenetic tree was reconstructed by the neighbor-joining (NJ) method (48).

Degradation experiment and metabolite identification. Cells of strain DY-1 were cultured in R2A medium for approximately 72 h at 30°C and then collected by centrifugation at $6,000 \times g$ for 5 min. The cell pellets were washed twice with sterilized MSM, adjusted to an optical density at 600 nm (OD_{600}) of approximately 1.5, and used as the inoculant. The standard degradation assay was performed in MSM containing 0.15 mM trifloxystrobin (pH 7.0) at 30°C with shaking (180 rpm). The initial inoculum of strain DY-1 cells was set at 1.15×10^7 CFU $\cdot$ ml $^{-1}$. The concentration of trifloxystrobin was determined by HPLC, and the metabolites were identified by HPLC-MS/MS. The effect of pH and temperature on the degradation of trifloxystrobin by strain DY-1 was investigated by the standard degradation assay at different pHs (pH 4.0 to 10.0, in increments of 1 pH unit) and incubation temperatures (15°C to 55°C, in increments of 5°C). The degradation of strobilurin fungicides, including azoxystrobin, picoxystrobin, and pyraclostrobin, by strain DY-1 was also studied using the standard degradation assay method. Each treatment was performed in triplicate, and the means and standard errors were calculated. The uninoculated medium was used as a control, and the change in the tested substrate concentration in the control was negligible.

Cloning of the strobilurin fungicide de-esterification gene *strH*. The shotgun method was used to clone the strobilurin fungicide de-esterification genes. The genomic DNA of strain DY-1 was extracted by the high-salt concentration precipitation method (49). The genomic DNA library of strain DY-1 was constructed as described by Wang et al. (36). Fractions containing approximately 2- to 6-kb fragments were recovered with a DNA gel extraction kit (Omega Bio-Tek Biotechnology Ltd., USA) and ligated into the pUC118 *Bam*HI/*BAP* plasmid (Takara). The ligation product was transformed into competent *E. coli* DH5 α cells, which were then plated on LB plates containing 100 mg $\cdot$ liter $^{-1}$ ampicillin and 0.15 mM trifloxystrobin and incubated at 37°C for approximately 24 h. The positive clones that produced clear transparent halos indicative of trifloxystrobin degradation were selected and further analyzed by HPLC analysis to detect the trifloxystrobin degradation ability. The inserted fragments in the recombinant plasmids harbored by the confirmed positive clones were sequenced by Nanjing GenScript Biotechnology Co., Ltd. (Nanjing, China). BLASTN and BLASTP (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) were used to analyze the nucleotide sequences and deduce the amino acids, respectively.

Gene expression and purification of the recombinant enzyme. The *strH* gene was amplified from the genomic DNA of strain DY-1 using primers StrH-F and StrH-R (Table 3). The amplicon was inserted into the *Nde*I and *Xho*I sites of pET-29a(+) to generate the recombinant plasmid pET-*strH*, which was then transformed to *E. coli* BL21(DE3). The transformants were subcultured into 100 ml LB medium and allowed to grow until the culture density reached 0.5 (OD_{600}). 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 8 min. The intact cells were removed by centrifugation at $13,000 \times g$ for 20 min (4°C). The StrH was purified on streptavidin-berpharose FF (Beijing Bersee Science and Technology Co., Ltd.). Protein concentration was determined by the Bradford method, with bovine serum albumin as the standard (43).

Enzyme activity assay. The standard enzyme activity assay of StrH was performed at 35°C for 10 min in 1 ml of 20 mM Tris-HCl buffer (pH 7.0) containing 0.24 mM trifloxystrobin and 3 μ g StrH. Later, the reaction was terminated by the addition of an equal volume of acetonitrile. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1.0 μ mol of substrate per min.

Enzyme kinetics was studied using different concentrations of trifloxystrobin (10 to 150 μ M) in the reaction mixture. The enzyme was diluted to 3 μ g to ensure that the consumption of the substrate was within the linear range during the reaction. The concentration of the 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. The enzyme kinetics analysis method for azoxystrobin, picoxystrobin, and pyraclostrobin was the same as for trifloxystrobin. All reactions were carried out in triplicate, and the data are reported as means $\pm$ standard deviations.

Biochemical properties of the recombinant StrH. The same concentration of trifloxystrobin as for the standard enzyme activity assay was used to investigate the optimal temperature and pH of StrH. StrH activity was investigated at temperatures between 20°C and 60°C (in increments of 10°C) to determine the optimal reaction temperature. Similarly, StrH was preincubated at various temperatures ranging from 20 to 60°C for 1 h to determine its thermal stability and residual activity. The nonheated enzyme was used as the control (100%). The optimal pH was measured at 35°C, with pHs ranging from 3.0 to 10.0. The buffers used were 20 mM citric acid-sodium citrate (pH 3.0 to 6.0), sodium phosphate (pH 6.0 to 8.0), and glycine-NaOH (pH 8.0 to 10.0) (in increments of 1 pH unit). StrH was incubated at 4°C for 1 h in different pH buffers to determine the pH stability and residual activity. The samples were collected before trifloxystrobin was completely consumed. The activity observed by the standard enzyme assay was defined as 100%, and the relative activities of each reaction mixture were calculated. StrH 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 7.0) to remove the EDTA and identify whether StrH is a metal-dependent enzyme. Enzyme activity was assayed as described above and compared with the StrH activity without EDTA treatment.

RNA isolation and quantitative real-time PCR. An aliquot of the cells of strain DY-1 was inoculated into 20 ml of MSM supplemented with 0.15 mM trifloxystrobin or 0.15 mM methanol, respectively, at a level of 2% (vol/vol). The cultures were incubated at 30°C for 10 h (about 50% of trifloxystrobin was transformed), and the cells were harvested by centrifugation ($6,000 \times g$, 5 min at 4°C). Total RNA was extracted using a MiniBEST universal RNA extraction kit (TaKaRa, China) and treated with a genomic

DNA (gDNA) eraser (TaKaRa), according to the manufacturer's instructions. A reverse transcription (RT) reaction was performed using a PrimeScript RT reagent kit (TaKaRa). Expression of *strH* in strain DY-1 was analyzed by the quantitative real-time PCR in an Applied Biosystems 7300 real-time PCR system (Applied Biosystems, USA) using an SYBR Premix Ex Taq RT-PCR kit (TaKaRa). The 16S rRNA gene was used as the internal control gene since it was transcribed in the presence and absence of trifloxystrobin, as demonstrated in the reverse transcription-PCR (data not shown). The gene-specific primers RT16S-F/R and RTSH-F/R used for quantitative real-time PCR are listed in Table 3. Relative changes in *strH* expression were calculated using the $2^{-\Delta\Delta CT}$ threshold cycle (C_T) number method (50).

Site-directed mutagenesis. Point mutations in StrH were constructed by overlap PCR. Primers StrH-F and StrH-R were used as the forward and reverse flanking primers, respectively. The internal primer pairs S232A-F/R, E354A-F/R, and H467A-F/R are listed in Table 3. All PCR assays were performed with the Phanta Max superfidelity DNA polymerase (Vazyme Biotech Co., Ltd., China) under a standard site-directed mutagenesis protocol (51). The PCR products were gel purified and then subsequently cloned into the NdeI and XhoI sites of the pET-29a(+) plasmid; successful substitutions were confirmed by DNA sequencing. Purification of the recombinant proteins and analysis of their activities against trifloxystrobin were performed as previously described.

Detoxification assay of strobilurin fungicides by strain DY-1. *Chlorella ellipsoidea*, a eukaryotic alga of freshwater, was used as an indicator to evaluate the toxicity of a given compound. After reaching the exponential phase, *C. ellipsoidea* and strain DY-1 were washed three times and used as inoculants. BG-11 medium was used in the detoxification assay (13, 14). The initial cell densities for *C. ellipsoidea* and strain DY-1 were set at 0.01 (OD_{680}) and 1.15×10^7 CFU $\cdot$ ml $^{-1}$. The strobilurin fungicides were added at a concentration of 1 mg $\cdot$ liter $^{-1}$. Since the addition of strain DY-1 had no effect on the growth of *C. ellipsoidea* in BG-11 medium (data not shown), three treatments were set as follows: T1 (*C. ellipsoidea* plus strobilurin fungicide), T2 (*C. ellipsoidea* plus strobilurin fungicide plus strain DY-1), and T3 (*C. ellipsoidea* plus strain DY-1). All cultures were cultivated in the above-mentioned microcosms and manually agitated three times a day. The cell density of *C. ellipsoidea* in the culture was measured every 24 h using a spectrophotometer at an OD_{680} (13, 14).

Analytical methods. The culture or enzyme assay samples were mixed with equal volumes of acetonitrile and then centrifuged at $13,000 \times g$ for 2 min to analyze the strobilurin fungicides and their metabolites. The mixtures were filtered through a 0.22- μ m Millipore membrane and used for HPLC analysis on an UltiMate 3000 titanium system (ThermoFisher Scientific) equipped with a C_{18} reversed-phase column (4.6 by 250 mm, 5 μ m; Agilent Technologies). The mobile phase was a mixture of acetonitrile, water, and acetic acid (75:24.5:0.5, vol/vol/vol) at a flow rate of 1.0 ml $\cdot$ min $^{-1}$. The column elution was monitored by measuring the absorbance at 220 nm. The injection volume was 20 μ l. The column temperature was maintained at 40°C. For identification of the intermediate metabolites, the mass spectrum was collected using a TripleTOF 5600 plus (AB SCIEX) mass spectrometer. The metabolites were ionized by electrospray ionization with positive polarity, and the characteristic fragment ions were detected using HPLC-MS/MS.

Data availability. The 16S rRNA gene sequence and the DNA fragment (3,247 bp) containing the esterase gene *strH* from strain DY-1 were deposited in the GenBank database under accession numbers MN625842 and MT992706, respectively. The GenBank accession number for the esterase gene *strH* of strain DY-1 is MT992707.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 5.7 MB.

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