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RESEARCH ARTICLE

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A new dimethylsulfoniopropionate lyase of the cupin superfamily in marine bacteria

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Abstract

Dimethylsulfoniopropionate (DMSP) is a marine organosulfur compound with important roles in stress protection, marine biogeochemical cycling, chemical signalling and atmospheric chemistry. Diverse marine microorganisms catabolize DMSP via DMSP lyases to generate the climate-cooling gas and info-chemical dimethyl sulphide. Abundant marine heterotrophs of the *Roseobacter* group (MRG) are well known for their ability to catabolize DMSP via diverse DMSP lyases. Here, a new DMSP lyase DddU within the MRG strain *Amylibacter cionae* H-12 and other related bacteria was identified. DddU is a cupin superfamily DMSP lyase like DddL, DddQ, DddW, DddK and DddY, but shares <15% amino acid sequence identity with these enzymes. Moreover, DddU proteins forms a distinct clade from these other cupin-containing DMSP lyases. Structural prediction and mutational analyses suggested that a conserved tyrosine residue is the key catalytic amino acid residue in DddU. Bioinformatic analysis indicated that the *dddU* gene, mainly from *Alphaproteobacteria*, is widely distributed in the Atlantic, Pacific, Indian and polar oceans. For reference, *dddU* is less abundant than *dddP*, *dddQ* and *dddK*, but much more frequent than *dddW*, *dddY* and *dddL* in marine environments. This study broadens our knowledge on the diversity of DMSP lyases, and enhances our understanding of marine DMSP biotransformation.

INTRODUCTION

The organosulfur compound dimethylsulfoniopropionate (DMSP) produced by marine phytoplankton, macroalgae, corals, angiosperms, animals and heterotrophic bacteria can reach several petagrams each year (Curson et al., 2017, 2018; Ksionzek et al., 2016; Otte, 2004; Raina et al., 2013; Stefels, 2000; Zhang et al., 2019). DMSP not only participates in the nutrient cycling to provide an important source of organic carbon and/or reduced sulphur for microbial communities (Kiene et al., 2000; Yoch, 2002), but also possesses important physiological functions that include, but are not limited to, acting as an osmolyte, cryoprotectant, predator deterrent, antioxidant, chemoattractant and protectant against high hydrostatic pressure (Cosquer et al., 1999; Karsten et al., 1996; Seymour et al., 2010; Sunda et al., 2002; Wolfe et al., 1997; Zheng et al., 2020). After DMSP is released into marine environment from the producer organisms during exudation, senescence, viral lysis, or grazer attack (Bratbak et al., 1995; Hill et al., 1998; Stefels & Boekel, 1993; Wolfe et al., 1997), it is degraded largely by bacteria (Yoch, 2002; Zhang et al., 2019).

To date, three DMSP metabolic pathways have been reported, the demethylation pathway, the oxidation pathway and the lysis pathway. The demethylation pathway is initiated by the DMSP demethylase enzyme DmdA, in which DMSP is finally catabolized to acetaldehyde and volatile methanethiol (MeSH) (Howard et al., 2006; Reisch et al., 2011). It is estimated that 50% to 90% of DMSP is catabolized through this pathway (Kiene et al., 2000). In the oxidation pathway, DMSP is first oxidized to dimethylsulfoxonium propionate (DMSOP) that can then be catabolized to dimethylsulfoxide (DMSO) and acrylate via unidentified DMSOP lyase enzymes (Thume et al., 2018). In the lysis pathway, DMSP is cleaved by diverse enzymes, including a DMSP CoA-transferase DddD (Todd et al., 2007), a DMSP-CoA ligase DddX (Li et al., 2021), and seven DMSP lyases (or DMSP dethiomethylases) (Alcolombri et al., 2015; Curson et al., 2008; Curson, Sullivan, et al., 2011; Sun et al., 2016; Todd et al., 2009, 2011, 2012), to generate the climate-active volatile dimethyl sulphide (DMS) and 3-hydroxypropionate-CoA (3-HP-CoA) (for DddD catalysis), acryloyl-CoA (for DddX catalysis) or acrylate (for DMSP lyases catalyses). DMS, which is generated with DMSP as its main precursor (de Souza & Yoch, 1995), is an important nutrient and an info-chemical in signalling pathways for diverse organisms (Nevitt, 2011; Shemi et al., 2021; Teng, Wang, et al., 2021). DMS represents the largest biogenic sulphur source entering the atmosphere (Andreae, 1990), and its oxidation products aid cloud formation and may influence weather and climate (Vallina & Simo, 2007). Acrylate, another product in the DMSP lysis pathway, can not only be utilized as a carbon source by some microorganisms (Curson, Todd, et al., 2011), but also possesses important physiological functions, for example, it protects marine bacteria from grazing by ciliate predators (Teng, Wang, et al., 2021). In marine bacteria, acrylate can be metabolized through the AcuN-AcuK pathway (Todd et al., 2010) and/or PrpE-AcuI pathway (Reisch et al., 2013).

Among the seven reported DMSP lyases, Alma1 is the only known algal DMSP lyase (Alcolombri et al., 2015), while the other six DMSP lyases (DddP, DddL, DddQ, DddW, DddY and DddK) were all identified from bacteria. Alma1 is a tetrameric, redox-sensitive enzyme of the aspartate racemase superfamily, yet its structure and catalytic mechanism is not elucidated (Alcolombri et al., 2015). DddP, existing in the marine *Roseobacter* group (MRG) (Simon et al., 2017), the SAR116 clade, *Gammaproteobacteria* and some ascomycete fungi (Curson, Todd, et al., 2011), is a member of the M24 metallopeptidase family (Todd et al., 2009), and the most abundant bacterial DMSP lyase in marine metagenomes database (Teng, Qin, et al., 2021). The crystal structure and the catalytic mechanism of DddP have been revealed (Hehemann et al., 2014; Wang et al., 2015). DMSP lyases DddL, DddQ, DddW, DddY and DddK all belong to the cupin superfamily, which comprises a functionally highly diverse group of proteins (Dunwell et al., 2004). DMSP lyases of the cupin superfamily share two conserved cupin motifs, and require divalent metal ions as cofactors for their enzymatic activities (Brummett et al., 2015; Li et al., 2014, 2017; Peng et al., 2019). Among them, DddL is membrane-associated (Teng, Wang, et al., 2021), DddY is periplasmic (Curson, Sullivan, et al., 2011), while the others are cytoplasmic. The biochemical properties, catalytic mechanism and ecological distribution for DddQ, DddY and DddK have been reported (Li et al., 2014, 2017; Peng et al., 2019). The biochemical properties of DddW have also been studied (Brummett et al., 2015). The *dddL*, *dddQ* and *dddW* genes mainly occur in MRG, while *dddK* is only found in SAR11 bacteria, and *dddY* is sporadically distributed in *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria* and *Epsilonproteobacteria* (Curson, Todd, et al., 2011). Recently, researchers found that many marine microorganisms that liberate DMS from DMSP do not contain known DMSP lyase genes in their genomes, suggesting that there are still more unrecognized DMSP lyases in the natural environments yet to be identified (Liu et al., 2018; Zhang et al., 2019).

In this study, *Amylibacter cionae* H-12, a member of the MRG (Wang et al., 2017), was found to exhibit DMSP-dependent DMS production (Ddd^{+}). However,

strain H-12 lacked known DMSP lyase genes in its genome sequence, indicating that this strain likely utilizes a novel enzyme to generate DMS from DMSP. Using genetic and biochemical approaches on strain H-12, a new cupin superfamily DMSP lyase, termed DddU, was discovered and characterized. Structural prediction and mutational work were done to investigate the DddU DMSP lysis mechanism and its key catalytic amino acid residues. Bioinformatic analysis was also conducted to study the environmental importance of DddU in comparison to the other known DMSP lyase genes. The results presented here broaden our knowledge on the diversity of DMSP lyases, and enhance our understanding of DMSP catabolism in marine environments.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Amylibacter cionae H-12, the Δ*dddU* mutant strain and the complemented Δ*dddU* strain (Δ*dddU*/pHGP$_{tac}$-*dddU*, Δ*dddU*/pHGP$_{tac}$) were cultured in the marine broth 2216 medium at 25°C, and kanamycin (Km) with a final concentration of 50 μg/mL was added into culture of complemented strain. The *E. coli* strains WM3064, DH5α and BL21 (DE3) were cultured in the Lysogeny Broth (LB) medium at 37°C, and diaminopimelic acid (DAP) with a final concentration of 0.3 mM was added into culture of *E. coli* WM3064. Strains were listed in Table S5.

Quantification of DMS by gas chromatography

Cells cultured in the marine broth 2216 medium to the late exponential phase were harvested, washed with sterilized artificial seawater three times and diluted to the $OD_{600} \approx 0.03$. Then the cells were incubated in the minimal medium supplied with 1 mM DMSP in gas-tight sealing bottles at 25°C for 3 h. The cultures were assayed for DMS production on gas chromatography (GC) (GC-2030, Shimadzu, Japan) equipped with a flame photometric detector according to the method described by Zhang (Zhang et al., 2014). The culture medium without bacteria and culture medium without DMSP were set as controls. A DMS standard was used as a positive control. A six-point calibration curve of DMS standards was used to quantify DMS production (Curson et al., 2017). Bacterial cells were lysed by ultrasonication, and proteins content in the cell extracts were measured by Pierce BCA Protein Assay Kit (Thermo, USA). DMS production is expressed as nmol DMS min^{-1} mg $protein^{-1}$.

Growth assay with DMSP as the sole carbon source

Cells were grown in the marine broth 2216 medium at 180 rpm and 25°C to the late exponential phase ($OD_{600} \approx 0.8$), and washed three times with sterilized artificial seawater. Then, 1% (v/v) cells were inoculated into the minimal medium with sodium pyruvate, DMSP or acrylate (5 mM) as the sole carbon source. Strains were cultured in the dark at 25°C and 180 rpm. The bacteria growths were measured by detecting the OD_{600} of the cultures using a spectrophotometer V-550 (Jasco Corporation, Japan).

Transcriptome sequencing of strain H-12

The strain H-12 was cultured in the marine broth 2216 medium at 180 rpm and 25°C to the late exponential phase ($OD_{600} \approx 0.8$). Subsequently, the cells were washed with sterilized artificial seawater three times and incubated in sterilized artificial seawater for 2 h. Then, cells were inoculated into the minimal medium with DMSP (5 mM) or with carbon mixture (1.11 g/L glucose, 1.01 g/L fructose, 1.38 g/L disodium succinate, 1.25 g/L sodium pyruvate, 1 mL/L glycerol, 1.39 g/L sodium acetate) as the carbon source for 4 h at 180 rpm and 25°C. Cells cultured in minimal medium with carbon mixture were set as control groups and with DMSP were set as experimental groups. Total RNA was extracted using a RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. After validating the quality, RNA samples were sent to Novogene Biotechnology Co., Ltd (China) for transcriptome sequencing and subsequent bioinformatic analyses.

Genetic manipulations of *Amylibacter cionae* H-12

Genomic DNA from strain H-12 was extracted using a bacterial genomic DNA isolation kit (BioTeke Corporation, China) according to the manufacturer's instructions. For in-frame deletion of the target gene *dddU*, the upstream 771-bp and downstream 816-bp flanking regions of *dddU* were amplified by PCR with the primer pairs *dddU*-5′O/*dddU*-5′I and *dddU*-3′I/*dddU*-3′O respectively, and cloned into pHGM01. The resulting plasmid was transformed into *E. coli* WM3064 and then mobilized into wild-type strain H-12 by conjugation as previously described (Fu et al., 2015). The *dddU* deletion mutant was obtained through homologous recombination.

For complementation of the Δ*dddU* mutant, the *dddU* gene was amplified using the primers set *dddU*-

pHGP$_{tac}$-F/*dddU*-pHGP$_{tac}$-R, and then inserted into pHGP$_{tac}$. The recombinant vector or empty vector was transformed into *E. coli* WM3064, and then mobilized into the Δ*dddU* mutant by conjugation, as detailed above. Colony PCR was used to confirm the presence of the transferred plasmid.

All PCR-amplified sequences were verified by DNA sequencing. The primers used were listed in Table S6.

Gene synthesis, point mutation, protein expression and purification

Full-length *dddU* gene, codon optimized for expression in *Escherichia coli*, was chemically synthesized and then subcloned into the pET-22b (Novagen, USA) by BGI gene Co., Ltd (China). The plasmid was also used as the template for site-directed mutagenesis. Point mutations in *dddU* were introduced using the PCR-based method and were verified by DNA sequencing. The primers used were listed in Table S6. The DddU protein and its enzyme variants were overexpressed in *E. coli* BL21 (DE3). The cells were cultured in the LB medium with 100 μg/mL ampicillin (Am) at 37°C to an OD_{600} of 0.8 ∼ 1.0 and then induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 18°C for 16 h. Briefly, cells were collected and resuspended in the lysis buffer (50 mM Tris–HCl, 100 mM NaCl, 0.5% glycerol, pH 8.0), and then lysed by cryogenic pressure crusher. The lysates were purified by affinity chromatography on a Ni^{2+}-nitrilotriacetic acid (NTA) column (GE healthcare, USA), and subsequently fractionated by gel filtration on a Superdex-G75 column (GE healthcare, USA). The purities of these recombinant proteins were analysed by SDS-PAGE, and protein concentrations were determined by the BCA assays.

Enzyme assay and characterization

The enzymatic activity of DddU was measured by detecting the production of acrylate as previously described (Wang et al., 2015). DddU (at a final concentration of 0.1 μM) and DMSP (at a final concentration of 5 mM) were mixed with reaction buffer containing 100 mM Tris–HCl (pH 7.0) in a total volume of 200 μL. After the mixture was incubated at 40°C for 10 min, the reaction was stopped by adding perchloric acid. The amount of acrylate in the reaction mixture was detected by high-performance liquid chromatography (HPLC) on a Sunfire C18 column (Waters, Ireland). The reaction mixture without DddU enzymes was set as the control group.

To determine the optimal temperature for *Pi*DddU, reaction mixtures were incubated at 0, 10, 20, 30, 40, 50 or 60°C for 10 min. The optimum pH for *Pi*DddU was examined at 40°C (the optimal temperature for *Pi*DddU enzymatic activity) using Britton-Robinson buffer at pH values of 4 to 10. The Britton–Robinson buffer is a mixture of 0.04 M H_3BO_3, 0.04 M H_3PO_4 and 0.04 M CH_3COOH (Barek et al., 1999). The kinetic parameters of *Pi*DddU were determined by adding *Pi*DddU (0.1 μM) into the reaction systems containing different concentrations of DMSP (1, 2, 3, 5, 10, 20, 25, 60 and 80 mM) under conditions of pH 7.0 at 40°C for 10 min, and then the non-linear analysis was performed based on the initial rates determined with different DMSP concentrations. The enzymatic activities of the *Pi*DddU enzyme variants were also examined under the optimum pH and temperature.

Circular dichroism spectroscopy assays

Circular dichroism (CD) spectra for wild-type *Pi*DddU and its enzyme variants were carried out in a 0.1 cm-path length cell on a JASCO J-1500 Spectrometer (Japan) at 25°C. All proteins were adjusted to a final concentration of 20 μM in 10 mM Tris–HCl (pH 8.0) and 100 mM NaCl. CD Spectra were recorded from 250 to 200 nm at a scan speed of 200 nm min^{-1}.

Bioinformatics

The Basic Local Alignment Search Tool (BLAST) was used to perform similarity searches and searched for sequences through the National Center for Biotechnology Information (NCBI) BLAST webpage interface (http://www.ncbi.nlm.nih.gov/BLAST/). DddU of *Amylibacter cionae* H-12 was used as the query sequence to search for homologues in the NCBI Reference Sequence Database using BLastP with the percentage identity >70%, and a total of 40 hits was retrieved. Among them, 28 sequences in genome-sequenced bacteria were selected for further phylogenetic analysis. Trees were constructed using the algorithm in the MEGA 7.0 software package. The distribution analyses of DddU homologues according to the method described by Teng (Teng, Qin, et al., 2021). Briefly, hidden Markov models (HMM) were created using protein sequences that are biochemically characterized. DddU homologues from metagenomes/metatranscriptomes were obtained using hmmsearch (http://hmmer.org). The cut-off value used was <e-30. The DddU homologues sequences retrieved from our bioinformatics pipeline were further scrutinized for the presence of conserved key residues involved in substrate binding or catalysis. The distribution of *dddU* homologues was analysed in metagenomic data from 60 polar seawater samples (NCBI BioProject accession no. PRJNA588686) and 174 non-polar *Tara* Ocean

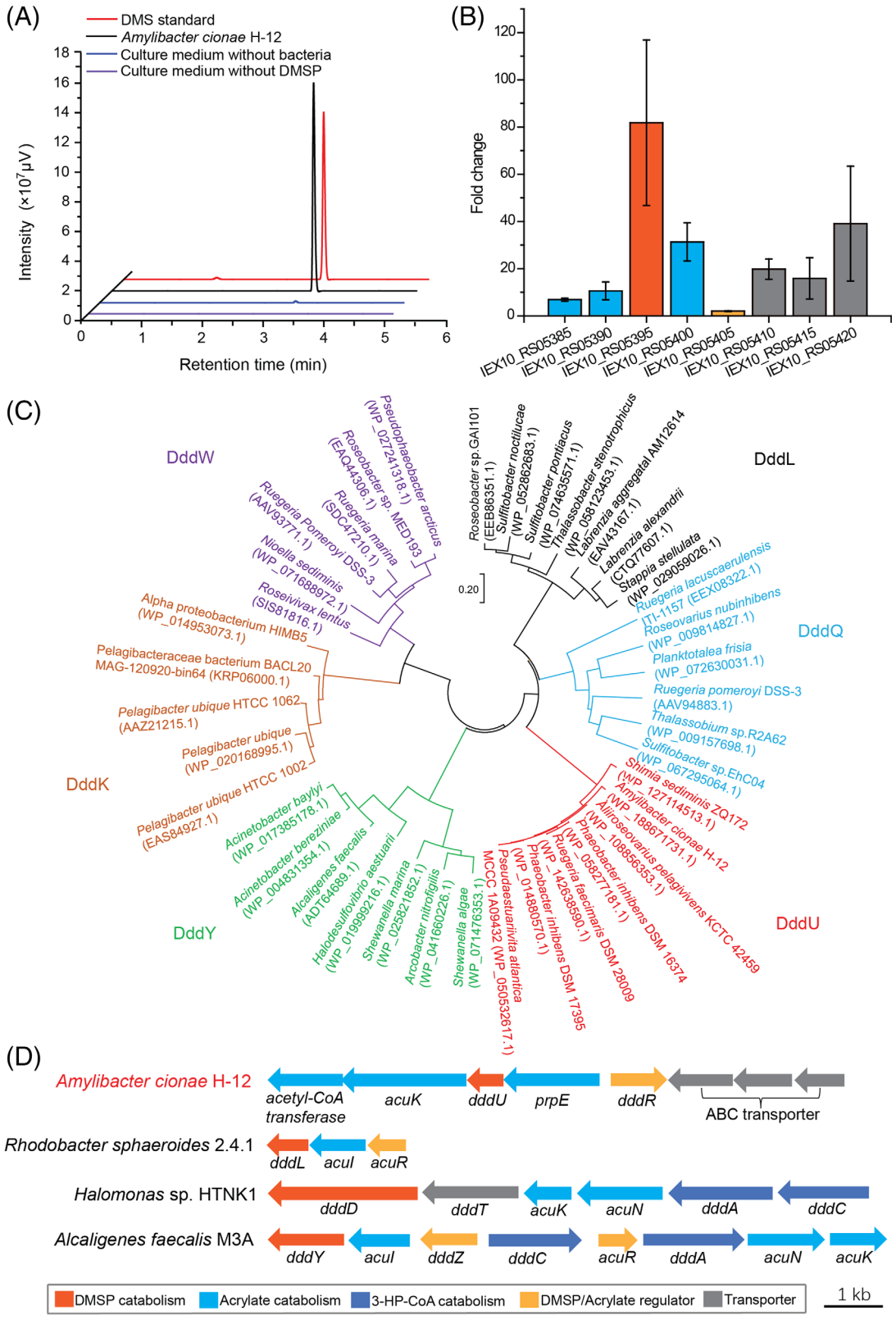

FIGURE 1 Analysis of *Amylibacter cionae* H-12 dimethylsulfoniopropionate (DMSP) lyase activity and its candidate DMSP lyase. (A) Gas chromatography detection of DMSP-dependent dimethyl sulphide (DMS) production by strain H-12. The culture medium without bacteria and the medium without DMSP were used as the controls. The DMS standard was used as a positive control. (B) Transcriptomic analysis of putative DMSP/acrylate-catabolizing genes from H-12. The fold changes were calculated by dividing the gene transcripts in the presence of 5 mM DMSP by those in the absence of DMSP. The error bar represents standard deviation of triplicate experiments. (C) Neighbour-joining phylogenetic tree of DddU and representative proteins of the five other reported cupin-containing DMSP lyases. Phylogenetic analysis was performed using MEGA version 7.0 (Tamura et al., 2013). (D) Genetic organization of the putative DMSP-catabolizing gene cluster in strain H-12. DMSP catabolic genes and their regulators from *Rhodobacter sphaeroides* 2.4.1, *Halomonas* sp. HTNK1 and *Alcaligenes faecalis* M3A were shown (Curson, Sullivan, et al., 2011).

samples (fraction size, 0.22–3 μm; http:// www.pangaea.de/). The amino acid sequences of 10 conserved bacterial marker genes (Sunagawa et al., 2013) were retrieved from the NCBI database, and the average abundance of these marker genes was used to normalize the abundance of the *dddU* gene as described previously (Curson et al., 2017), that is, the relative abundance of *dddU* in each site is calculated by the ratio of *dddU* abundance to the average abundance of 10 selected bacterial marker genes. The distribution of *dddU* transcripts was analysed in *Tara* Oceans Microbiome Reference Gene Catalogue v2+ meta T Arctic Inside (prokaryotes) dataset from *Tara* Ocean samples (https://tara-oceans.mio.osupytheas.fr/ocean-gene-atlas/) and used the percent of mapped reads to express the relative abundance. The geographical distributions of *dddU* and its transcripts were constructed by Ocean Data View.

RESULTS AND DISCUSSION

Discovery of a novel DMSP lyase in a marine *Roseobacter* strain

Amylibacter cionae H-12 was isolated from a sea squirt *Ciona savignyi* collected from Jiaozhou Bay, China (Wang et al., 2017). Through routine screening of available marine bacteria by GC analysis, the strain H-12 was found to be able to catabolize DMSP and produce DMS (485 ± 62 nmol DMS min^{-1} mg $protein^{-1}$) in the minimal medium supplemented with DMSP (Figure 1A), although it was unable to utilize DMSP as a sole carbon source (Figure S1).

To identify the DMSP lyase enzymes in strain H-12, the sequenced genome (GenBank accession number: GCA_014643735.1) was searched for gene products homologous to DddD, DddP, DddQ, DddX, DddY, DddW, DddL, DddK and Alma1. However, no proteins homologous to these enzymes with amino acid sequence identity >25% were found (Table S1), implying that H-12 may possess novel DMSP lyase enzyme(s). The transcriptomes of strain H-12 in the presence and absence of 5 mM DMSP were then sequenced. Transcriptomic analysis showed that the transcripts of eight genes that compose a gene cluster were highly upregulated (Figure 1B; Table S2) when strain H-12 was induced by DMSP.

In the gene cluster, IEX10_RS05385 was annotated as an acetyl-CoA C-acyltransferase. Although no significant amino acid sequence identity was found between IEX10_RS05385 and AcuN (Todd et al., 2010), they both belong to the CoA-transferase family III. IEX10_RS05390 was annotated as an enoyl-CoA hydratase, sharing 32% amino acid sequence identity with AcuK in *Halomonas* sp. HTNK1 (Todd et al., 2010). IEX10_RS05395 was annotated as a DMSP lyase family protein, however, the amino acid sequence of this protein possesses <15% identity to any reported DMSP lyases. IEX10_RS05400 was annotated as a fatty acid-CoA ligase, and shared 25% sequence identity with PrpE in *Ruegeria pomeroyi* DSS-3 (Reisch et al., 2013). AcuN, AcuK and PrpE were reported to participate in acrylate catabolism (Reisch et al., 2013; Todd et al., 2010). IEX10_RS05405 was annotated as a LysR family transcriptional regulator, sharing 30% sequence identity with DddR in *Pseudomonas* sp. J465 (Curson et al., 2010). IEX10_RS05410, IEX10_RS05415 and IEX10_RS05420 constituted an ABC (ATP-binding cassette)-type transporter, which may play an important role in importing DMSP for strain H-12 (Li et al., 2023). The results above suggested that the gene cluster IEX10_RS05385-IEX10_RS05420 in strain H-12 is highly possible to be involved in DMSP catabolism, and IEX10_RS05395 may encode a new DMSP lyase, which we term as DddU hereafter. Sequence analysis showed that the DddU protein contained two conserved cupin motifs (Dunwell et al., 2004), and thus belongs to the cupin superfamily. Neighbour-joining phylogenetic analysis revealed that DddU was most closely related to DddQ but it clearly formed a separate clade to DddQ and the other known cupin-containing DMSP lyases (Figure 1C), suggesting the divergent evolution of DddU from other cupin DMSP lyases. Among reported DMSP lyase genes, *dddL*, *dddD* and *dddY* were found in the *ddd-acu* cluster (Figure 1D). The gene *dddU* also locates in the *ddd-acu* cluster. However, the pattern of the DMSP-catabolizing cluster in strain H-12 is different from the patterns of those reported DMSP-catabolizing gene clusters (Figure 1D).

Functional verification of DddU

To confirm that *dddU* encoded a DMSP lyase in strain H-12, a Δ*dddU* mutant strain was constructed in which the majority of this gene was deleted by homologous recombination (Figure S2A). The H-12 Δ*dddU* mutant strain was shown produce ~2-fold less DMS from DMSP compared wild type H-12 and this reduction in DMSP lyase activity was fully complemented back to wild type levels by cloned *dddU* on $pHGP_{tac}$-*dddU* (Figure 2A; Figure S2B). These data are consistent with *dddU* encoding an enzyme with in vivo DMSP lyase activity. Given that the Δ*dddU* mutation did not completely abolish the DMSP lyase activity of strain H-12, this strain likely contains other unidentified DMSP lyase(s). The phenomenon of multiple DMSP lyases co-occurring in the same host is very common, particularly in MRG bacteria (Teng, Qin, et al., 2021). For

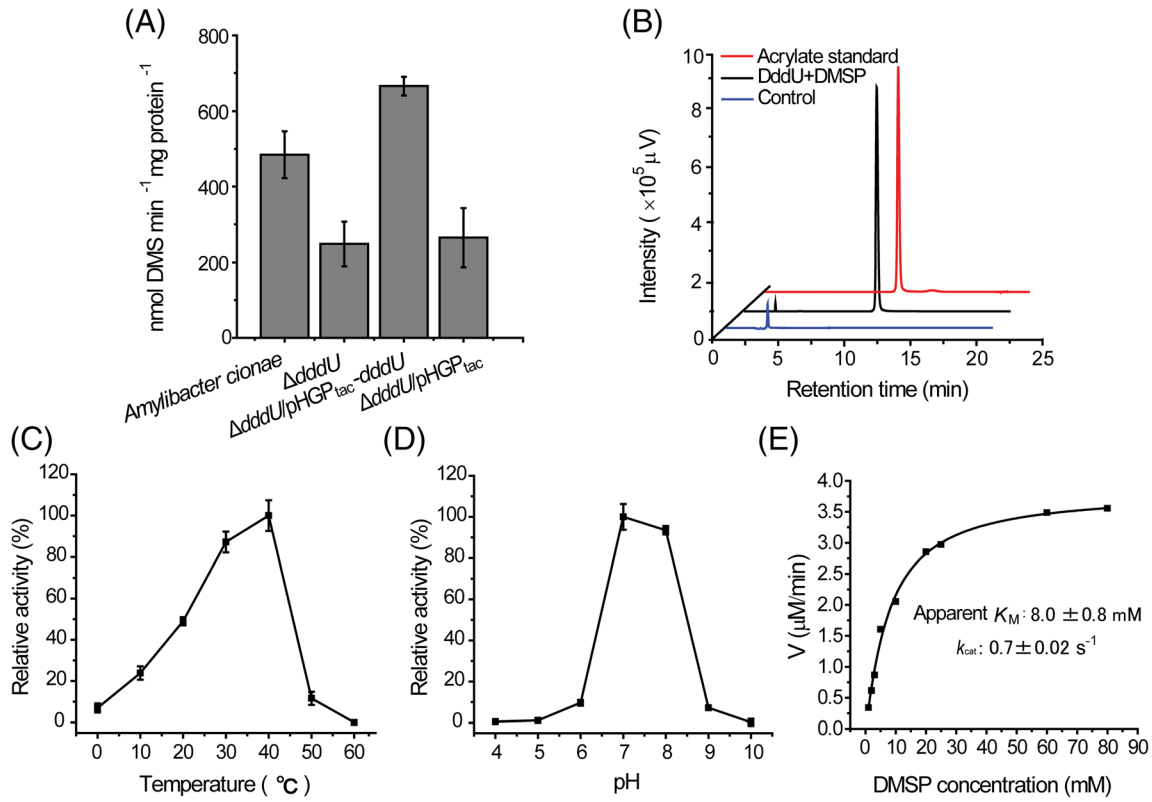

FIGURE 2 DddU is a dimethylsulfoniopropionate (DMSP) lyase. (A) The DMSP-dependent dimethyl sulphide production from the wild-type strain *Amylibacter cionae*, the Δ*dddU* mutant, the complemented mutant Δ*dddU*/pHGP$_{tac}$-*dddU*, and the mutant containing an empty vector Δ*dddU*/pHGP$_{tac}$. The error bar represents standard deviation of triplicate experiments. (B) High-performance liquid chromatography detection of the acrylate produced from the cleavage of DMSP by recombinant DddU. The reaction system without DddU was used as the control. The acrylate standard was used as a positive control. (C) Effect of temperature on the enzymatic activity of *Pi*DddU. The error bar represents standard deviation of triplicate experiments. (D) Effect of pH on the enzymatic activity of *Pi*DddU. The error bar represents standard deviation of triplicate experiments. (E) Nonlinear fit curves for DMSP cleavage by *Pi*DddU. The kinetic parameters were determined under pH 7.0 at 40°C.

example, *Ruegeria pomeroyi* DSS-3 possesses DddP, DddW and DddQ, and *Roseovarius nubinhibens* ISM contains DddP and DddQ. Why a bacterium possesses multiple DMSP lyases is unknown, but may be linked to potentially different roles for DMSP catabolism in an organism, for example a DMSP lyase for growth on DMSP and one for signalling processes that may be differentially regulated.

Expression and characterization of DddU

To demonstrate in vitro DMSP lyase activity for H-12 DddU, full-length *dddU*, codon optimized for expression in *Escherichia coli*, was chemically synthesized, overexpressed in *E. coli* BL21 (DE3) cells, and the recombinant DddU was purified.

HPLC analysis showed that DddU catalysed the release of acrylate from DMSP (Figure 2B; Table S3), confirming that DddU was a functional DMSP lyase in vitro. However, the enzymatic activity of DddU from H-12 is relatively weak (0.15 μM acrylate min^{-1} μM protein^{-1}; Table S3). A DddU homologue from *Phaeobacter inhibens* P66 (*Pi*DddU), sharing 93% sequence identity with H-12 DddU, exhibited much higher activity towards DMSP (5.03 μM acrylate min^{-1} μM protein^{-1}; Table S3), and was selected for further characterization. The optimal temperature for *Pi*DddU enzymatic activity was 40°C (Figure 2C), and the optimal pH was 7.0 (Figure 2D). This optimal temperature for *Pi*DddU was relatively high compared to that in most marine environments. However, *Pi*DddU maintained ~50% of its highest enzymatic activity at 20°C and ~85% at 30°C (Figure 2C), indicating that *Pi*DddU is a viable enzyme in physiological environments.

The recombinant *Pi*DddU exhibited a K_M value of 8.0 mM for DMSP at pH 7.0 and 40°C (Figure 2E), which is lower than that of the reported DMSP lyases DddP, DddQ, DddW and Alma1, but higher than that of DddY and DddK (Table 1). These relatively high K_M values at the millimolar level are common in DMSP lyases and may help bacteria maintain intracellular DMSP at in vivo levels to perform physiological functions, for example, in stress protection. The k_{cat} of *Pi*DddU for DMSP was 0.7 s^{-1} (Figure 2E), lower than that of most other reported DMSP lyases (Table 1).

TABLE 1 Kinetic parameters of different dimethylsulfoniopropionate (DMSP) lyases towards DMSP.

Protein	Organism	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	References
*Pi*DddU	*Phaeobacter inhibens* P66	8.0 ± 0.8	0.7 ± 0.02	87.8	This study
DddY	*Acinetobacter bereziniae*	5.0 ± 0.6	8.3 ± 0.5 × 10^3	1.7 × 10^6	Li et al. (2017)
DddP	*Roseovarius nubinhibens* ISM	13.8 ± 5.5	0.3 ± 0.1	18.7	Kirkwood et al. (2010)
	Phaeobacter inhibens DSM 17395	34.8 ± 4.7	3.9 ± 0.18	1.1 × 10^2	Burkhardt et al. (2017)
DddQ	*Ruegeria lacuscaerulensis* ITI_1157	21.5 ± 6.8	1.0 ± 0.3	46.5	Li et al. (2014)
	Ruegeria pomeroyi DSS-3	28.6 ± 3.3	0.9 ± 0.04	32	Burkhardt et al. (2017)
DddW	*Ruegeria pomeroyi* DSS-3	8.7 ± 0.7	18.3	2.1 × 10^3	Brummett et al. (2015)
	Ruegeria pomeroyi DSS-3	12.8 ± 0.8	16.8 ± 0.4	1.3 × 10^3	Burkhardt et al. (2017)
DddK	*Pelagibacter ubique* HTCC 1062	3.7 ± 0.6	0.9 ± 0.1	2.4 × 10^2	Peng et al. (2019)
Alma1	*Emiliania huxleyi*	9.0 ± 0.9	7.0 ± 0.3 × 10^2	7.8 × 10^4	Alcolombri et al. (2015)

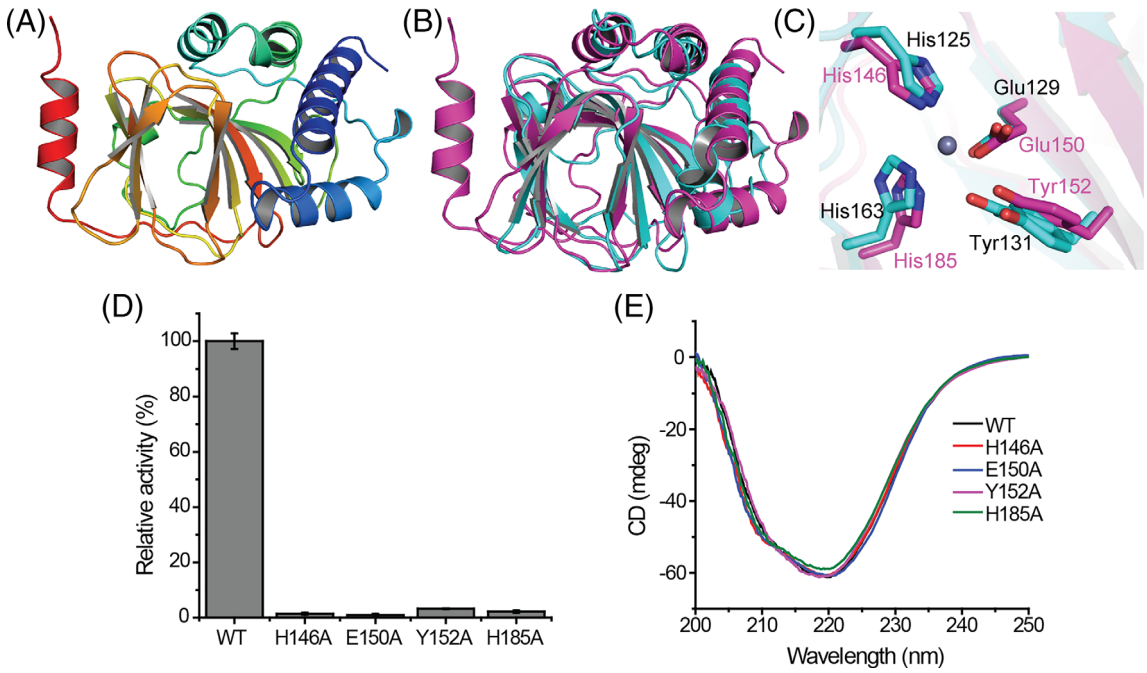

FIGURE 3 Structural and mutational analyses of *Pi*DddU. (A) The predicted structure of *Pi*DddU. (B) Alignment of the *Pi*DddU (purple) and DddQ (cyan, PDB ID: 4LA2) protein structures. (C) Key residues predicted to be involved in *Pi*DddU dimethylsulfoniopropionate (DMSP) cleavage. Residues of *Pi*DddU are coloured in purple, and of DddQ in cyan. (D) DMSP lyase activity of *Pi*DddU amino acid substitution mutants. The activity of native *Pi*DddU was defined as 100%. The error bar represents standard deviation of triplicate experiments. (E) Circular dichroism (CD) spectra of native *Pi*DddU and its enzyme variants. CD spectra of the proteins at a final concentration of approximately 20 μM were collected from 250 to 200 nm.

Key residues involved in DddU catalysis

To further investigate the catalytic mechanism of *Pi*DddU DMSP lysis, we tried to crystallize *Pi*DddU, but unfortunately all attempts failed. Instead, the structure of *Pi*DddU was predicted using AlphaFold2 (Jumper et al., 2021). The *Pi*DddU structure was predicted to contain a β-barrel fold surrounded by several α-helices (Figure 3A). Despite the low amino acid sequence identity between *Pi*DddU and DddQ (∼13%), their structures were similar (Figure 3B), with a root mean square deviation of 1.8 Å over 104 C_α atoms between these two structures. Moreover, structural alignment of *Pi*DddU and DddQ indicated that residues involved in metal ion binding (His146, Glu150, Tyr152 and His185 in *Pi*DddU) and catalysis (Tyr152 in *Pi*DddU) were perfectly superposed (Figure 3C), implying that *Pi*DddU utilizes a similar catalytic mechanism to DddQ (Li et al., 2014), with Tyr152 being the catalytic residue to initiate the β-elimination reaction. Substitution of these His146, Glu150, Tyr152 and His185 residues to alanine almost abolished the enzymatic activity of *Pi*DddU (Figure 3D), supporting the important roles of these residues. Furthermore, CD spectroscopy analysis showed that the secondary structures of the enzyme variants exhibited little deviation from that of wild-type

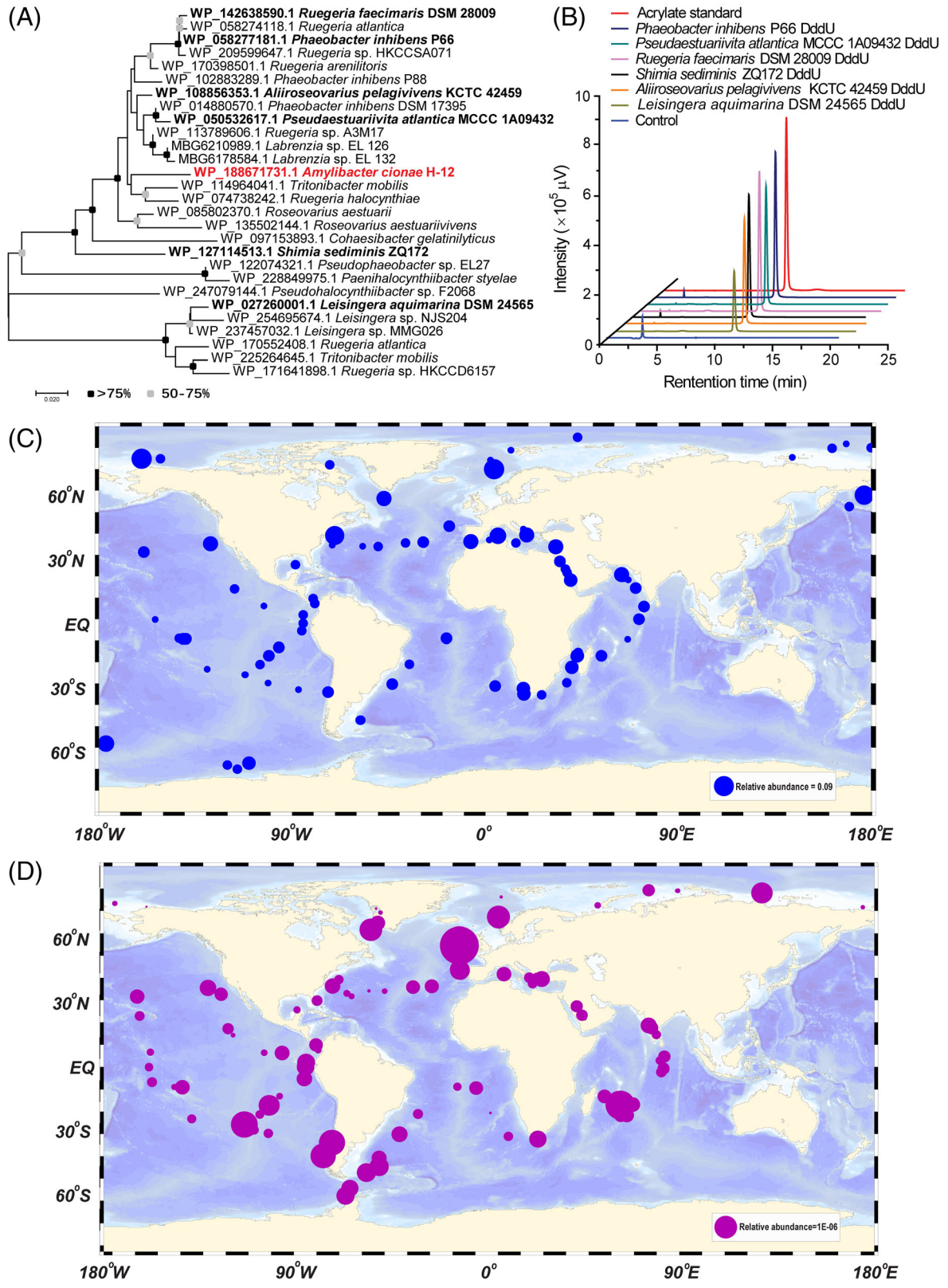

FIGURE 4 Distribution of DddU in bacteria. (A) The phylogenetic tree of DddU enzymes in *Alphaproteobacteria*. The black dots represented >75% of the bootstrap values and grey dots represented 50%–75%. Phylogenetic analysis was performed using MEGA version 7.0 (Tamura et al., 2013). Those DddU homologues that are functionally characterized are highlighted in bold. (B) Detection of the dimethylsulfoniopropionate cleavage activity of DddU homologues via high-performance liquid chromatography. The reaction system without DddU enzymes was used as the control. The acrylate standard was used as a positive control. (C) The geographic distribution of *dddU* (blue symbols). The relative abundance of *dddU* at each station was represented by different sizes symbols. (D) The geographic distribution of *dddU* transcripts (purple symbols). The relative abundance of *dddU* transcripts at each station was represented by different sizes symbols.

(WT) *Pi*DddU (Figure 3E), indicating that the loss of DMSP lyase activity was due to amino acid replacement rather than any major structural change.

Distribution of DddU in bacteria

The distribution of *dddU* in sequenced genomes available on the NCBI Reference Sequence database was analysed to predict those with DMSP lyase activity. DddU proteins (with >70% amino acid sequence identity to H-12 DddU) were predicted in many *Alphaproteobacteria*, most being MRG (Figure 4A). Multiple sequence alignment showed that the key amino acid residues His146, Glu 150, Tyr152 and His185 were highly conserved (Figure S3), suggesting that these candidates DddU were likely functional DMSP lyases. Indeed, several of these representative candidate DddU proteins were expressed and purified from *E. coli* BL21 (DE3) and all exhibited DMSP lyase activity (Figure 4B; Table S3), implying that bacteria expressing this enzyme would cleave DMSP.

To infer the environmental importance of *dddU* compared with other bacterial DMSP lyase genes, the relative abundance of these genes in metagenomic data from polar waters (60 samples) and non-polar *Tara* Ocean (174 samples) was assayed. The relative abundance of DddU homologues is lower than DddP, DddQ and DddK but higher than DddW, DddY, and DddL (Table S4; Figure S4). In addition, *dddU* and its transcripts widely dispersed among the marine metagenomic and metatranscriptomic samples in the Atlantic, Pacific, Indian and polar oceans (Figure 4C,D), suggesting that *dddU* may play an important role in the global sulphur cycling.

CONCLUSION

DMSP is ubiquitous in marine environments, and its cleavage produces the climate-active gas DMS. In this study, a new DMSP lyase DddU from an MRG strain *Amylibacter cionae* H-12 was identified. DddU is a cupin superfamily DMSP lyase that is phylogenetically distinct to other reported cupin-containing DMSP lyases. Despite the lack of sequence similarity, DddU likely possesses a very similar catalytic mechanism with DddQ. *dddU* exists in many *Alphaproteobacteria*, particularly MRG, and is widely distributed in the global oceans. This study broadens our knowledge on the diversity of DMSP lyases, and enhances our understanding of the DMSP biotransformation in marine environments.

AUTHOR CONTRIBUTIONS

Shu-Yan Wang: Formal analysis (lead); investigation (lead); writing – original draft (lead). **Nan Zhang:** Formal analysis (equal); investigation (equal). **Zhao-Jie Teng:** Data curation (lead); formal analysis (equal). **Xiao-Di Wang:** Data curation (equal); formal analysis (equal). **Jonathan D. Todd:** Writing – review and editing (equal). **Yu-Zhong Zhang:** Conceptualization (equal); funding acquisition (equal); supervision (supporting). **Hai-Yan Cao:** Formal analysis (equal); writing – original draft (supporting); writing – review and editing (supporting). **Chun-Yang Li:** Conceptualization (lead); funding acquisition (lead); supervision (lead); writing – review and editing (lead).

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the figures, table and supporting information of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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