The Capability of Utilizing Abiotic Enantiomers of Amino Acids by *Halomonas* sp. LMO_D1 Derived From the Mariana Trench

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D-amino acids (D-AAs) have been produced both in organisms and in environments via biotic or abiotic processes. However, the existence of these organic materials and associated microbial degradation activity has not been previously investigated in subduction zones where tectonic activities result in the release of hydrothermal organic matter. Here, we isolated the bacterium *Halomonas* sp. LMO_D1 from a sample obtained from the Mariana trench, and we determined that this isolate utilized 13 different D-AAs (D-Ala, D-Glu, D-Asp, D-Ser, D-Leu, D-Val, D-Tyr, D-Gln, D-Asn, D-Pro, D-Arg, D-Phe, and D-Ile) in the laboratory and could grow on D-AAs under high hydrostatic pressure (HHP). Moreover, the metabolism of L-AAs was more severely impaired under HHP conditions compared with that of their enantiomers. The essential function gene (Chr_2344) required for D-AA catabolism in strain LMO_D1 was identified and confirmed according to the fosmid library method used on the D-AAs plate. The encoded enzyme of this gene (DAADH_2344) was identified as D-amino acid dehydrogenase (DAADH), and this gene product supports the catabolism of a broad range of D-AAs. The ubiquitous distribution of DAADHs within the Mariana Trench sediments suggests that microorganisms that utilize D-AAs are common within these sediments. Our findings provide novel insights into the microbial potential for utilizing abiotic enantiomers of amino acids within the subduction zone of the Mariana trench under HHP, and our results provide an instructive significance for understanding these abiotic enantiomers and allow for insights regarding how organisms within extraterrestrial HHP environments can potentially cope with toxic D-AAs.

Keywords: D-amino acids, Mariana trench, high hydrostatic pressure, D-amino acids dehydrogenase, abiotic enantiomers

INTRODUCTION

Among all three domains of life, proteins are composed of 19 L-form amino acids (along with the achiral glycine), and these are known as the 20 proteinogenic amino acids (Burton and Berger, 2018). The use of one type of enantiomers instead of enantiomers mixtures is considered to be the foundation for the stability of biomolecules (Hein and Blackmond, 2012). Although single handedness has been selected for use in proteins, both of the mirror-image forms of amino acids are stable and available in nature.
acids can be generated via abiotic or biotic processes. The simulated scenarios in the context of laboratory abiotic synthesis of these chiral molecules, including spark discharge experiments (Miller, 1953), the Strecker reaction (Peltzer et al., 1984), and other hydrothermal reactions (Kebukawa et al., 2017) have demonstrated that racemic mixtures are always yielded. Racemization is another non-negligible process that can generate enantiomer mixtures. The estimated racemization half-lives of amino acids are 0.1–1 million years at temperatures comparable with that of the surface of the Earth (Bada and Miller, 1987). Natural abiotic synthesis products are primarily identified in meteorites, where the vast majority of chiral molecules are racemic (Pizzarello, 2006). To date, 14 of the 20 proteinogenic amino acids (Ala, Asp, Glu, Gly, Ile, Leu, Pro, Ser, Thr, Val, Tyr, Phe, Met, and Trp) have been detected in meteorites or identified in abiotic synthesis (Menez et al., 2018; Glavin et al., 2020). The amounts of amino acids can reach the order of ppm (parts per million) and can vary according to the types of amino acids or meteorites (Pizzarello et al., 2012; Glavin et al., 2020). In addition to their generation via biotic processes, certain D-AAs have also been discovered in some functional structures present in prokaryotes, eukaryotes, and viruses. For example, D-AAs (usually D-Ala, D-Glu, D-Ser, and D-Asp) are components of the cell walls of bacteria (Vollmer et al., 2008). D-Ser acts as a neurotransmitter in the brain (Snyder and Kim, 2000). These D-AAs are generated from L-AAs by enzymatic racemization that occurs via racemases (Zhang and Sun, 2014) and are released into the environment by direct secretion or through cell lysis (Zhang et al., 2015).

As enantiomers of proteinogenic amino acids, D-AAs present within various environments are toxic to organisms due to their ability to interfere with translation by binding to tRNA (Soutourina et al., 2004) and to interfere with the formation of peptidoglycans (Caparros et al., 1992). Therefore, the catabolism of D-AAs is a significant component of the detoxification process, and this process can be summarized into two aspects that include the direct oxidation of D-AAs to α-keto acids and the transformation of D-AAs into L-enantiomers in a manner that is coupled with L-AA catabolism (Zhang et al., 2021). Related enzymes involved in D-AA catabolism can be divided into broad-spectrum oxidases/dehydrogenases, substrate-specific oxidases, and racemases. DAADHs are the most commonly used broad-spectrum oxidases for D-AA direct oxidation to the corresponding α-keto acids and ammonia in prokaryotes, and have been identified in various species such as Pseudomonas aeruginosa (Marshall and Sokatch, 1968), Escherichia coli (Olsiewski et al., 1980), and Helicobacter pylori (Tanigawa et al., 2010). D-AA oxidases catalyze the oxidative deamination of uncharged aliphatic, aromatic, and polar D-AAs to produce α-keto acids, ammonia, and hydrogen peroxide that have been observed in most eukaryotic organisms and in some bacteria (Pollegioni et al., 2008). The substrate-specific oxidases include D-aspartate oxidase, D-Ser dehydratase, D-Cys desulphydrase, and D-proline dehydrogenase. Conversely, amino acid racemases that function to mediate the interconversion of amino acid enantiomers are ubiquitous in bacteria and are consequently considered to play an important role in preventing the toxicity of D-AAs (Zhang and Sun, 2014). In archaea, a PLP-dependent racemase enables Pyrococcus horikoshii OT-3 to offset the essential L-amino acid requirement by adding D-type enantiomers (Kawakami et al., 2015). These enzymes are related to either D-AA oxidation or to D-AA transformation to the L-enantiomers and can also enable the utilization of D-AAs as carbon or nitrogen sources. The bioavailability of D-AAs has been verified in a number of microbes that have been isolated from a number of sources including soils, the rhizosphere (Radkov et al., 2016), seawater, and sediments (Fu et al., 2016; Kubota et al., 2016; Yu et al., 2019). As the formation of D-AAs by biotic racemization is a ubiquitous process, the utilization of D-AAs may not be limited to the above ecosystems. In this study, we primarily aim to extend the knowledge of the catabolic processes of D-AAs to the deepest area on Earth (the hadal trench).

The hadal trench possesses a water depth of greater than 6,000 m and contains complex processes that occur at different scales, such as geology, physical dynamics, geochemistry, and biology, and this is currently the least studied area within the ocean (Jamieson, 2011). The subduction zones in the hadal trench was formed as a result of oceanic plate converging with continental plate in combination with multiple extreme characteristics, such as extremely deep water depth, high pressure, cold, and geological activity. Organic matter arrived in the hadal trench through deposition from upper water or due to migration from steep slopes that benefit from the unique funnel-like shape of the trench and frequent seismic activity (Jamieson, 2011; Luo et al., 2017). During the descent of the oceanic plate, materials within the subduction slab and the seawater are transported to the overlying mantle (Plumper et al., 2017) or reach the oceanic mantle through cracks generated by slab flexing (Zhou et al., 2015). The slab hydration and serpentinitization processes liberated mantle carbon and, by reducing power, generated alkaline fluids that erupted through slab faults and fueled the ecosystem (Ohara et al., 2012). Evidence of abiotically formatted amino acids beneath the Atlantis Massif during the alteration of serpentinites indicate the potential for fluid–rock interactions that may allow for abiotic formation of amino acids in the oceanic lithosphere (Menez et al., 2018). No available data have indicated the existence of D-AAs within the Mariana trench; however, it is still reasonable to assume that both abiotic and biotic racemization from L-AAs and also the abiotic formation of amino acids can generate D-AAs within the hadal environment.

Here, we report a bacterial strain (Halomonas sp. LMO_D1) that possesses the ability to utilize D-AAs, and this bacterial strain was enriched and isolated from subduction zones within the Mariana trench. Cultivation experiments demonstrated that the LMO_D1 strain could grow on D-AAs even under HHP conditions. One of the essential enzymes for D-AA utilization (DAADH) has been confirmed to function well both in vivo and in vitro. Our results revealed the capability of one microbe to utilize D-AAs under HHP conditions within the subduction zone, and these results extended our knowledge of the microbes within...
the hadal trench and provided a valuable microbiological material for abiotic enantiomer utilization.

**MATERIALS AND METHODS**

**Sample collection**

The sediment sample MT086 from the Mariana trench was used in this study for enrichment and strain isolation. MT086 was collected in the Challenger Deep (11°11.6988′N, 141°48.7008′E) at a depth of 8,636 m during cruise TS01 using a submersible manipulator in August of 2016. The collected samples were stored in sterile pressurized vessels at 45 MPa and 4°C for further processing.

**Enrichment, isolation, and cultivation using defined medium**

The medium used in this study was based on an artificial seawater (ASW) and composed of the following components per liter: 26.0 g of NaCl, 5.0 g of MgCl2·6H2O, 1.06 g of CaCl2·2H2O, 4.0 g of Na2SO4, 0.3 g of NH4Cl, 0.1 g of KH2PO4, 0.5 of g KCl, and 2.52 g of NaHCO3. Additionally, ASW was supplemented with 1 ml of a trace element mixture, a vitamin mixture, a vitamin B12 solution, and a thiamine solution as described previously (Widdel et al., 2006). The pH of the medium was adjusted to 7.0 using 1 M HCl solution. A mixture of 19 D-AAs (0.01% w/v for each) was supplemented as the sole carbon source for enrichment and isolation of microbes with corresponding metabolic potential. The cultures were incubated at 8°C, and this was designed based on the optimal growth temperature of the majority of obligate piezophiles (2°C–10°C). To test the bioavailability of different amino acids, the cells were harvested, washed three times with PBS buffer, and then inoculated into ASW medium that was supplemented with 6 mM amino acids. When amino acids served as the sole nitrogen source, NH4Cl was removed, and 6 mM glucose was added.

Enrichment and cultivation under various pressures were both performed as previously described (Wang et al., 2021). Sediments (0.15% w/v) or pure-cultured cells (initial OD600 = 0.01) were inoculated into the defined medium. The cultures were transferred into sterile plastic syringes and sealed with a rubber cover. The syringes were placed into a stainless pressure vessel (Feiyu Science and Technology Exploitation Co. Ltd., Nantong, China) and compressed as described in a previous study (Wang et al., 2021). Each culture was repeated in triplicate.

**DNA extraction and PCR/qPCR amplification of 16S rRNA genes**

Total DNA from sediments and enrichment samples was extracted and purified using the FastDNA™ Spin Kit for Soil (MP Biomedicals, USA) following the manual protocol. Genomic DNA from strain LMO_D1 was extracted using the phenol–chloroform method. The full-length bacterial 16S rRNA genes were amplified using primers 27F (5-AGAGTTTGATCCTGGGCTCAG-3) and 1492R (5-GGTTACCTTGTCTACAG-3). The amplified products were ligated into the pMD18-T vector (TaKaRa, Tokyo, Japan) for sequencing. To determine the cell density of the sediment samples and enrichment culture, 16S rRNA genes from bacteria and archaea were quantified by qPCR using primers 341F/519R (5-GGTTACCCAGGGCTGTG3-3/5-ACGCAGCGGTAAAAGAAGG-3) and 519F/906R (5-CAGCMCGCGGCGGTA-3/5-CGGACCAATTCCTTTAGGT-3), respectively. Plasmids possessing archaea and bacterial 16S rRNA genes were used for standard curve construction. The quantification standard consisted of a dilution series of 1 × 102 and 1 × 106 copies/μl. To analyze the microbial composition, the V4 and V4–V5 regions of the 16S rRNA genes were amplified with primers 515F (5-GTGCACCMGCGCGGTAA-3) and 806R (5-GGACTACHVGGGTWTCTAAT-3) for bacteria and 516F (5-TGYAGCCGCGGGTAAHACCGVC-3) and 855R (5-TCCCGCCAATTCTTTAATA-3) for archaea.

**Genome and 16S rRNA gene amplicon sequencing and data analysis**

Genomic DNA was sequenced using an Illumina HiSeq (400 bp pair-end library; Illumina, USA) and PacBio (20-kb insert library; Pacific Biosciences, USA) platform at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). The raw sequence reads were trimmed with SOAPec v2.0 based on the k-mer length at 17 (Luo et al., 2012). The trimmed sequences from the Illumina HiSeq platform were assembled using A5-miseq version 20160825 (Tritt et al., 2012) and SPAdes genome assembler v3.11.1 (Bankevich et al., 2012). Sequences from the PacBio platform were assembled using HGA4 (Chin et al., 2016) and CANU v1.6 (Koren et al., 2017). Misassemblies were detected using MUMmer v3 (Delcher et al., 1999) and corrected using Pilon version 1.22 (Walker et al., 2014). Open reading frames (ORFs) were predicted using GeneMarkS version 4.32 (Besemer et al., 2001). The annotation and alignment of ORFs were both performed using Diamond v0.9.10.111 (Buchfink et al., 2015) against the NCBI NR database using an E-value cutoff of less than 10–6.

The 16S rRNA gene amplicons with different barcodes were mixed together at the same amounts and sequenced on an Illumina MiSeq platform using 2 × 250-bp cycles. For raw sequence data, low-quality reads (average quality value <20) and lengths <150 bp and also any ambiguities were excluded from the analysis. Further analysis was performed using the QIME2 version 2020.11 software pipeline (Bolyen et al., 2019). Taxonomic assignment was conducted using the SILVA database release 138 (Quast et al., 2013).

**Fosmid library construction, screening, and identification**

The genomic library of the LMO_D1 strain was constructed using the CopyControl™ HTP Fosmid Library Production Kit (Cat. No. CGFOS059) according to the instructions of the manufacturer. High-molecular-weight genomic DNA was sheared randomly, ligated into the fosmid vector pCC2FOS™,
and transfected into *E. coli* strain EPI300<sup>TM</sup>-T1<sup>R</sup>. Then clones were inoculated onto M9 minimal agar that was supplemented with a D-AAs mixture (0.01% w/v for each), 12.5 μg ml<sup>-1</sup> of chloramphenicol, and 0.1% CopyControl<sup>TM</sup> induction solution to induce clones with high copy numbers. Fosmid DNA from positive clones was extracted using the E. Z.N.A.<sup>™</sup> plasmid mini kit (Omega Bio-Tek, USA). The flanking sequences of the cloned insert DNA were sequenced using pCC2FOS forward and reverse sequencing primers (5′-GTACAACGA CACCTAGAC-3′ and 5′-CAGGAAACAGCTAGGA-3′) and blasted with the genome of strain LMO_D1 to identify the position of the insert DNA within the genome.

**Protein expression, purification, and enzyme assay**

The DAADH gene (Chr_2344) was cloned into the pET-28a vector between the Ndel and HindIII sites and expressed heterologously in *E. coli* BL21 (DE3). The recombinant *E. coli* strain was induced with isopropyl-β-D-thiogalactoside (0.1 mM) at 16°C for 20 h. Cells were harvested by centrifugation at 3,000 × g for 3 min and suspended in Buffer A containing 20 mM Tris-HCl (pH 8.0), 0.3 M NaCl, and 10% glycerol. Cells were lysed by sonication, and cell debris was removed by centrifugation at 8,000 × g for 40 min. The soluble and membrane fractions were separated by ultra-speed centrifugation at 160,000 × g for 60 min. The membrane fraction was suspended in buffer A that was supplemented with 0.03% Triton X-100. Sonication and ultra-speed centrifugation were repeated to obtain solubilized membrane proteins. The target protein was purified using Ni<sup>2+</sup>-NTA resin and eluted with Buffer B containing 20 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 0.3 M imidazole, and 10% glycerol.

Enzyme activity was detected using a modified method as described previously (Tanigawa et al., 2010). The reaction mixture was composed of 50 mM Tris-HCl (pH 8.0), 20 mM D-AA, 10 μM flavin adenine dinucleotide (FAD), 1.8 μM phenazine methosulfate (PMS), 0.5 mM 2,6-dichlorophenolindophenol (DCIP), and an appropriate amount of enzyme. The reaction was initiated by the addition of substrate, subsequently performed at 37°C, and ultimately terminated by the addition of 0.25% SDS. Enzyme activity was quantified by measuring the reduction of DCIP at 600 nm. As PMS could be reduced nonenzymatically by Cys, the substrate specificity for Cys was detected using the 3-methyl-2-benzothiazolinone hydrazine (MBTH) method as described previously (Yu et al., 2019).

**RESULTS**

**Enriching and screening of a microorganism possessing D-amino acid metabolic potential**

We conducted two sets of enrichment experiments that were supplemented with D-AAs as the sole carbon source. Each set of enrichment experiments was performed using two independent groups that differed in regard to cultivation pressure, and these groups were denoted as GA (at 0.1 MPa) and GH (at 70 MPa). As low temperature and HHP resulted in a reduced growth rate, the enrichment was continued for 69 days. After enrichment, the biomass yields of the GA and GH groups were estimated by quantification of 16S rRNA that was increased to 4.87 × 10<sup>8</sup> and 2.61 × 10<sup>5</sup> cells/ml, respectively, from an initial biomass of 1.0 × 10<sup>3</sup> cells/ml. The structures of the microbial communities for these two enrichments were analyzed and compared with that of the original sediment via 16S rRNA amplicon analysis (Materials and methods section). The enrichment samples were dominated by *Halomonas*, and the abundances of this species were increased to 95.5% and 73.9% in the GA and GH groups from 1.9% in the sediment sample MT086. The abundance of *Macellibacteroides* was also increased to 0.9% and 11.7% in the GA and GH groups, and this value was less than 0.001% in MT086 (Figure 1).

Strain isolation was performed by spreading sediment or enrichment samples onto the plates. The growth was
monitored, and visible clones were present after a 2-week incubation at 8°C. When the screening plates were supplemented with all 19 D-AAs as the sole carbon source, strain LMO_D1 derived from the sediment sample MT086 was identified as *Halomonas* (Figure 1B). Strains were also isolated from D-AA-enriched samples of the GA and GH groups, and these were all identified as the same species as strain LMO_D1.

**D-amino acid utilization by strain LMO_D1 under atmospheric pressure**

When supplied as nutrients, D-AAs can be utilized as carbon or nitrogen sources, and these were separately tested in this study. The capacity of strain LMO_D1 to utilize each individual D-AA as a sole carbon and/or sole nitrogen source was investigated by cultivation under atmospheric pressure (0.1 MPa) (Figure 2A). For comparison, 20 proteinogenic amino acids (L-type) were also used as the sole carbon/nitrogen sources to measure the growth of the LMO_D1 strain. Each individual amino acid at a concentration of 6 mM was tested as the sole nitrogen source (6 mM D-glucose supplemented as a carbon source). Modified ASW medium (NH4Cl removed) without the addition of any nitrogen source was used as a negative control. Individually, all 19 D-AAs were able to independently support the growth of strain LMO_D1, as indicated by an obviously increasing biomass compared with that of the negative control. Similarly, 18L-AAs and Gly could also be utilized as a nitrogen source by strain LMO_D1, with the exception of L-Cys. L-Cys cannot support the growth of strain LMO_D1, while D-Cys does support the growth of this strain. For the other 18 amino acids where both the L and D-types can be used as sole nitrogen sources, an overall improved growth was observed in response to supplementation with L-AAs compared with that observed in response to supplementation with D-AAs, and this was particularly true for Phe, His, Lys, Glu, Asp, Gln, and Asn (Figure 2).

When each individual amino acid at a concentration of 6 mM was tested as a sole carbon source (5.6 mM NH4Cl was used as the nitrogen source), ASW medium without the addition of any carbon source was used as a negative control. Strain LMO_D1 exhibited broad utilization capability for these D-AAs (13 individuals in total), including four common D-AAs (D-Ala, D-Glu, D-Asp, and D-Ser) that are components of peptidoglycans in most bacteria and have been widely reported to be present in marine systems (McCarthy et al., 1998) and nine uncommon D-AAs (D-Leu, D-Val, D-Tyr, D-Gln, D-Asn, D-Pro, D-Arg, D-Phe, D-Ile) that are rarely detected in environments...
Utilization of D-amino acids by strain LMO_D1 under high hydrostatic pressure

Considering that strain LMO_D1 was isolated from the hadal zone with a water depth of 8,636 m (equal to ~86 MPa) where the HHP caused by in situ water depth typically affects the metabolism of microorganisms, we also conducted D-AA utilization experiments under HHP to test if D-AA utilization can occur in the in situ environment (Figure 2B). The enrichment results indicated that the utilization of D-AAs occurred under 70 MPa conditions. However, the pre-experiments revealed that it is difficult to monitor and quantify the growth in a relatively short time under 70 and 110 MPa conditions, despite the integral cells being observed under these two pressures (Figure 2C). Thus, we chose a moderate pressure of 50 MPa that represents an upper limit that inhibits the growth of most piezotolerant microorganisms (Bartlett, 2002) to perform physiological experiments under HHP.

Strain LMO_D1 was then cultivated by supplementing individual L- or D-AAs as sole carbon sources under 50 MPa, and these were compared with cultivations performed under 0.1 MPa (Figure 2B). In general, the growth yields under HHP were lower than those under 0.1 MPa, and this was consistent with the enrichment results. When supplemented with D-AAs, growth under two different pressures exhibited a similar trend during the early stage. Subsequently, the maximum biomass yields were observed after approximately 9 and 13 days of incubation under 50 and 0.1 MPa, respectively, with a 2.52-fold higher biomass yield under 0.1 MPa. However, when cultivated with L-AAs, strain LMO_D1 exhibited a significantly higher growth rate and biomass yield under 0.1 MPa compared with these values in response to cultivation under 50 MPa. The maximum biomass under 0.1 MPa was 4.29-fold higher than that under 50 MPa. When comparing the cultivation results in response to supplementation with L-AAs and D-AAs, better growth was observed under both 0.1 and 50 MPa when L-AAs were supplemented. The growth variances under two different pressures were more significant when L-AAs were supplemented, and this indicated that the influence of HHP was different for the utilization of different amino acid enantiomers. In our experimental conditions, L-AA metabolism was more severely impaired under HHP compared with this value using their enantiomers.

Genomic evidence of D-amino acid catabolism

To investigate the catabolism of D-AA, the complete genome of Halomonas sp. LMO_D1 was acquired using Illumina Hiseq and Pacbio platforms and subsequently analyzed (Materials and methods section ). The complete genome of strain LMO_D1 contained a circular chromosome possessing a sequence length of 5.41 Mb and GC content of 54.77%. The genome possessed the highest identity with Halomonas sp. KHS3 (average nucleotide identity = 99.12% with 91.57% sequence alignment) that was isolated from the seawater of Mar del Plata. This bacterial strain harbors a strong ability to degrade different types of aromatic compounds (Corti Monzon et al., 2018), and no reports have been published on the capacity of this strain to utilize D-AAs.

According to the genomic information, strain LMO_D1 contained a complete glycolysis/glycogenesis pathway and TCA cycle that enabled the catabolism of the carbon skeleton of D-AAs. Four types of D-AA catabolism genes were identified in the strain LMO_D1 genome, including eight amino acid racemases, four DAADHs, one D-Ser dehydratase, and one D-Cys desulphhydrase (Table 1). Racemase mediates the interconversion of enantiomers. The annotated amino acid racemases present in strain LMO_D1 included alanine racemase, glutamate racemase, Asp/Glu racemase, and proline racemase. DAADHs are FAD-dependent oxidases that catalyze the deamination of D-AAs to the corresponding α-keto acids and ammonia. Four genes (Chr_2344, Chr_3502, Chr_3902, and Chr_4236) were annotated as DAADH within the genome of strain LMO_D1 based on the KEGG and COG databases (Galperin et al., 2021; Kanehisa et al., 2021). However, the proteins encoded by these genes shared very low identity

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**TABLE 1 | Annotated genes involved in D-AAs metabolism.**

| Classification | Gene ID | Annotation |
|----------------|---------|------------|
| Racemase       | Chr_1049| Glutamate racemase |
|                | Chr_1066| Proline racemase |
|                | Chr_1375| Asp/Glu racemase |
|                | Chr_1541| Glutamate racemase |
|                | Chr_3379| Proline racemase |
|                | Chr_3588| Alanine racemase |
|                | Chr_3707| Asp/Glu/hydantoin racemase |
|                | Chr_4336| Asp/Glu racemase |
| Dehydrogenase  | Chr_1066| D-amino acid dehydrogenase (Phenylalanine) |
|                | Chr_3502| D-amino acid dehydrogenase (Phenylalanine) |
|                | Chr_4236| D-amino acid dehydrogenase (Phenylalanine) |
|                | Chr_3902| D-amino acid dehydrogenase, small subunit |
| Dehydratase    | Chr_1770| D-serine dehydratase |
| Desulphhydrase | Chr_4292| D-cysteine desulphhydrase |
TABLE 2 | Substrate specificity of DAADH_2344.

| Substrate    | Enzyme activity (µmol min⁻¹ mg⁻¹) | (%)  |
|--------------|-----------------------------------|------|
| D-Methionine | 2.20 ± 0.02                       | 100  |
| D-Phenylalanine | 2.18 ± 0.07                     | 99.2 |
| D-Histidine  | 1.23 ± 0.02                       | 56.2 |
| D-Valine     | 1.22 ± 0.08                       | 55.7 |
| D-Leucine    | 1.14 ± 0.02                       | 52.1 |
| D-Tyrosine   | 1.13 ± 0.02                       | 51.2 |
| D-Tryptophan | 0.74 ± 0.10                       | 33.8 |
| D-Threonine  | 0.69 ± 0.01                       | 31.3 |
| D-Isoleucine | 0.57 ± 0.01                       | 26.2 |
| D-Alanine    | 0.33 ± 0.01                       | 14.8 |
| D-Asparagine | 0.31 ± 0.01                       | 14.1 |
| D-Serine     | 0.23 ± 0.01                       | 10.5 |
| D-Arginine   | 0.17 ± 0.01                       | 7.6  |
| D-Glutamine  | 0.12 ± 0.01                       | 5.6  |
| D-Aspartic acid | 0.10                      | 4.7  |
| D-Lysine     | 0.03 ± 0.02                       | 1.4  |
| D-Glutamic acid | --                        | --   |
| D-Proline    | --                                | --   |
| D-Cysteine   | --                                | --   |

Functional gene screening for D-amino acid catabolism via fosmid library

To further identify key enzymes functioning in D-AA metabolism, a genomic DNA fosmid library of strain LMO_D1 in the heterologous host E. coli EPI300™-T1R was screened based on functions, where target clones grew on a mixture of 19 D-AAs as the sole carbon source with NH₄Cl as the nitrogen source. The host cells were confirmed to be unable to subsist on D-AAs. The titer of the constructed library was estimated to be 50,000 colony-forming units (cfu) per milliliter. The number of clones required to ensure a 99% probability of a given DNA sequence for strain LMO_D1 (genome = 5.4 Mb) contained within a fosmid library composed of 30-kb inserts was calculated as N = ln (1–0.99)/ln [1–(30 × 10⁶ bases/5.4 × 10⁶ bases)] = 827 clones. A total of 3,168 clones (containing ~95.0 Mb sequence) from the library were inoculated on the D-AA screening plate, and 13 clones grew on the plate after 72-h incubation (Supplementary Figure S1).

The sequencing results revealed that there is a 9,203-bp sequence (the location in the genome is 2,544,984–2,554,187) containing seven coding sequences (Supplementary Figure S2) that are mutual for the 13 positive clones. Among them, five coding sequences were annotated with a definite function, and only one of them (Chr_2344) was annotated as a D-AA dehydrogenase (DAADH_2344) that may be related to D-AA utilization. The fosmid DNA of all 13 positive clones covered an overall 64.4-kb sequence (the location in the genome is 2,515,951–2,579,915) containing the coding sequences from Chr_2310 to Chr_2371. With the exception of the encoding gene for DAADH_2344 (Chr_2344), none of the other genes were annotated as related genes for D-AA metabolism. Specifically, the clones containing DAADH_2344 were able to grow on the D-AAs plate; other genes involved in D-AA catabolism were not included in the insert DNA of the positive clones. Our findings revealed a key role for DAADH_2344 in D-AA utilization in strain LMO_D1.

DAADH_2344 exhibited low identity (~28.34%) with all reviewed DAADHs according to the Swiss-Protein database (UniProt, 2021) (Supplementary Figure S3). Among the DAADHs whose functions have been identified, DAADH_2344 possessed the highest identity (26.00%) with DAADH from Pseudomonas aeruginosa PAO1 (NCBI accession NP_253,991.1). These low identities compared with the known DAADH indicate that the function of DAADH_2344 should be further confirmed.

Purification and identification of key enzyme D-amino acid dehydrogenase_2344 for D-amino acid metabolism

To further identify the function of DAADH_2344, we expressed its encoding gene (Chr_2344) in recombinant E. coli. Although DAADH has been reported as a membrane-bond protein in previous studies (Olsiewski et al., 1980; Tanigawa et al., 2010), we observed that DAADH_2344 was primarily distributed in the soluble fraction, thus, indicating that DAADH_2344 is a novel type DAADH compared with the previously reported enzyme (Supplementary Figure S4). The apparent molecular mass of the purified enzyme possessing two copies of the His6-tag was approximately 47 kDa according to SDS-PAGE, and this was consistent with the calculated value of 47.27 kDa for DAADH_2344 (Supplementary Figure S4).

The purified DAADH_2344 exhibited the specific activity of this enzyme in the presence of cofactor FAD and possessed a broad substrate scope according to the results of substrate specificity measurements using 19 D-AAs. In total, DAADH_2344 exhibited enzymatic activity against 16 D-AAs. By measuring the reduction of DCIP, the highest specific activity was observed for D-Met and D-Phe with specific activities of 2.20 and 2.18 µmol min⁻¹ mg⁻¹, respectively, and this was followed by D-His, D-Val, and D-Leu with ~50% specific activity compared with that against D-Met. D-Trp, D-Thr, and D-Ile with ~30% specific activity compared with that against D-Met. Relatively low activities were observed for D-Ala, D-Asn, D-Ser, D-Arg, D-Gln, D-Asp, and D-Lys (~15% compared with D-Met). In contrast, no activity was observed for D-Pro, D-Glu, and D-Cys (Table 2). Additionally, DAADH_2344 exhibited strict stereospecificity, and none of the L-AAs could be used as a substrate.

Interestingly, despite the ability of the enzyme DAADH_2344 to function well against D-Met, D-Trp, D-Thr, and D-His in vitro, the strain LMO_D1 can only utilize D-Met, D-Trp, D-Thr, and D-His physiologically (the same as their L-type) as a nitrogen source.
source and not as a carbon source. The failure to utilize these amino acids was assumed to be restricted by the catabolism of corresponding \(\alpha\)-keto acids (4-methylthio-2-oxobutanoic acid, indolepyruvate, 2-oxobutanoate, urocanate), and the pathways for these compounds to enter the TCA cycle were incomplete in the genome of strain LMO_D1. Thus, neither the D nor the L-type of these amino acids was bioavailable as a carbon source. In contrast, strain LMO_D1 could utilize D-Pro, D-Glu, and D-Cys as carbon or nitrogen sources for which DAADH exhibited no activity. Hence, the catabolism of D-Pro, D-Glu, and D-Cys may be conducted by other D-AA catabolic enzymes, such as racemase, desulhydrase, or other DAADHs encoded by the LMO_D1 strain (Table 1). The other 11 D-AAs (D-Ala, D-Asp, D-Ser, D-Leu, D-Val, D-Tyr, D-Gln, D-Asn, D-Arg, D-Phe, and D-Ile) that can support the growth of strain LMO_D1 could be deaminated by DAADH_2344. Based on the genomic evidence, the deamination product of 3 D-AAs [pyruvate (D-Ala and D-Ser) and oxaloacetate (D-Asp)] could enter the TCA cycle directly. Among the \(\alpha\)-keto acids of other D-AAs, phenylpyruvate (D-Phe), 4-methyl-2-oxopentanoate (D-Leu), and (S)-3-Methyl-2-oxopentanoic acid (D-Ile) could be transformed into acetyl-CoA, 4-hydroxyphenylpyruvate (D-Tyr) could be transformed to fumarate, and 3-methyl-2-oxobutanoic acid (D-Val) could be transformed to succinate. The 5-guanidino-2-oxopentanoate (D-Arg) could be transformed to L-Arg via L-arginine:pyruvate aminotransferase, and this could later be catabolized to succinate. The 2-oxoglutarate (D-Gln) and 2-oxosuccinamate could be transformed to 2-oxoglutarate and oxaloacetate, respectively, via the function of omega-amidase.

### DISCUSSION

In this study, we isolated *Halomonas* sp. LMO_D1 that possesses a broad range of D-AA utilization capability from the sample...
collected from subduction zones in the Mariana trench. In previous studies, the catabolism of D-AAs was primarily examined in soils, surface marine zones, or sediments within a 1,500-m water depth (Kubota et al., 2016). Our study extends the examined in soils, surface marine zones, or sediments within a previous studies, the catabolism of D-AAs was primarily collected from subduction zones in the Mariana trench. In Wang et al. D-Amino Acids Utilization in Mariana Trench Halomonas (Kawasaki and Benner, 2006; Vranova et al., 2011). Therefore, the capability of D-AA catabolism by Halomonas sp. LMO_D1 indicated a potentially high abundance of D-AAs in the hadal trench. Additionally, the hadal trench is an oligotrophic environment in which the catabolism of D-AAs is a strategy for better use of limited nutrients.

The isolate in this study, Halomonas sp. LMO_D1 is the reported microorganism that can utilize the broadest range of D-AAs. When comparing the bioavailability of D-AAs and L-AAs, the usable D-AAs for strain LMO_D1 are less abundant than are L-AAs as carbon sources, and this indicates that the carbon skeleton degradation of D-AAs shared a similar pathway to that of their L-enantiomer. However, when D-AAs are utilized as a nitrogen source, one more D-AA (D-Cys) can support cell growth, while their L-enantiomers cannot. In the genome of strain LMO_D1, the existence of D-Cys desulfhydrase that converts D-Cys into hydrogen sul exhibits a greater preference for D-AAs that are less common in peptidoglycans and instead are likely generated through abiotic processes or other biotic processes such as secretions by microorganisms for physiological regulation (Lam et al., 2009).

Based on the various production mechanisms of D-AAs within the environment, we believe that D-AAs exert an impact on the hadal trench ecosystem. However, we only obtained Halomonas in the enrichment and isolation experiments. The substrate concentration supplied in this study (100 mg/L of each D-AA) was much higher than the concentration of free D-AAs in the environment (Lomstein et al., 2006), and this may have resulted in a selective growth advantage for the dominant group and the inhibition of the growth of microbes possessing lower tolerances to D-AAs. As two catabolic strategies for coping with D-AAs, racemases are universal in bacteria and represent a common adaptation to the toxic D-AA environment. Moreover, oxidase/dehydrogenases allow microorganisms to possess an enhanced ability to utilize D-AAs. We attempted to fully understand these microorganisms based on metagenomic data. DAADHs are widespread in all samples examined in this study, and this indicates that D-AA utilizers are common in the environment. D-AA-utilizing species are likely far more diverse than the limited species that we isolated in our current study. In addition to the limitations of our isolation methods, some amino acid auxotrophs may be able to utilize D-AAs as a carbon/nitrogen source but not as their sole source. Additionally, we can search for more D-AA utilizers based on metagenome data. The discovery of an increasing abundance of DAADH in conjunction with sediment depth is consistent with the increasing proportion of D-AAs with sediment depth that was reported in a previous study (Pedersen et al., 2001), likely due to the observation that D-AAs were retained for a longer period compared with L-AAs and also likely due to the racemization process. As the generation of D-AAs is mediated by both biotic and abiotic processes, more sample sites are required to clarify the connection between the abundance of DAADH and the water depth of samples and the contribution of hydrothermal activities in regard to D-AA input in the subduction zone.

In this study, the cultivation experiments under HHP demonstrated that the strain was able to perform D-AA utilization in the original environment. The enrichment experiment was performed at 70 MPa, and the increased biomass suggested that D-AA utilization can occur at least at a water depth of 7,000 m. Under these conditions, the growth of most mesophilic microorganisms, such as E. coli, from the surface is completely inhibited (Bartlett, 2002). The strain LMO_D1
continued to grow at such a high hydrostatic pressure, and this indicated that it performed as a piezotolerant bacterium that should not originate from surface water. We observed that HHP exerted different degrees of impact on the metabolism of amino acid enantiomers. Under HHP, the metabolism of L-AAs was impaired more severely than that of D-AAs. A likely reason for this is that DAADH mediated the catabolic reaction using quinone rather than oxygen as an electron acceptor, thus, avoiding the production of H$_2$O$_2$ that would diminish cellular oxidative stress and benefit growth under HHP (Xiao et al., 2021).

The phenomenon of HHP in reducing the difference between L-AAs and D-AAs in regard to supporting growth is common or exclusive in strain LMO_D1 required further investigation in other deep-sea inhabitants. The study of how piezotolerant or piezophilic life copes with toxic D-AAs here on Earth will inspire speculation regarding how extraterrestrial life will inspire speculation regarding how extraterrestrial life. The expanded knowledge regarding the different physiological characteristics required in response to L-AAs and D-AAs will aid in refining life detection experiments (Navarro-Gonzalez et al., 2003; Zhang et al., 2021).

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA744311 and https://www.ncbi.nlm.nih.gov/.

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