JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Single-Cell-Based Screening and Engineering of D-Amino Acid Amidohydrolases Using Artificial Amidophenol Substrates and Microbial Biosensors

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Cite This: *J. Agric. Food Chem.* 2022, 70, 1203−1211 Read Online

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ABSTRACT: Enantiomerically pure D-amino acids are important intermediates as chiral building blocks for peptidomimetics and semisynthetic antibiotics. Here, a transcriptional factor-based screening strategy was used for the rapid screening of D-stereospecific amino acid amidase via an enzyme-specific amidophenol substrate. We used a D-threonine amidophenyl derivative to produce 2-aminophenol that serves as a putative enzyme indicator in the presence of D-threonine amidases. Comparative analyses of known bacterial species indicated that several *Bacillus* strains produce amidase and form putative indicators in culture media. The estimated amidase was cloned and subjected to rapid directed evolution through biosensor cells. Consequently, we characterized the F119A mutation that significantly improved the catalytic activity toward D-alanine, D-threonine, and D-glutamate. Its beneficial effects were confirmed by higher conversions and recurrent applications of the mutant enzyme, compared to the wild-type. This study showed that rapid directed evolution with biosensors coupled to designed substrates is useful to develop biocatalytic processes.

KEYWORDS: *amidase, D-amino acid, artificial substrate, biosensor, genetic circuit, high-throughput screening*

INTRODUCTION

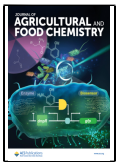
Enantiomerically pure D-amino acids are chiral building blocks for pharmaceuticals, food additives, herbicides, and agrochemicals.[1] D-*p*-Hydroxyphenylglycine and D-phenylglycine are used to synthesize penicillin derivatives.[2] D-Valine, D-aspartic acid, D-threonine, D-phenylalanine, D-serine, D-tryptophan, and D-proline are applied in the syntheses of antibiotics and other drugs.[3−5] D-Alanine is a starting material for the artificial sweetener alitame.[6] D-Threonine is used as a precursor for several important antibiotics, including cefbuperazone as a cephalosporine-type second-generation antibiotic, himastatin with antitumor activity, and teixobactin as a Gram-positive bacterial antibiotic.[7] Though D-amino acid production is important, there are insufficient highly active enzymes available to manufacture them. It is also difficult to obtain optically pure D-amino acids at high yield from racemic mixtures.

Several methods can be used to prepare pure chiral D-amino acids through chemical synthesis or enzymatic resolution methods using inexpensively manufactured D,L-racemic amino acid derivatives.[8−10] Among them, the D-amino acid amidase-based method is one of the most promising approaches because of the easy racemization of the remaining L-configurations of amino acid derivatives.[11,12] D-amino acid amidase (DaaA) hydrolyzes the carboxyl amide bonds of D-amino acid amides to form D-amino acids and ammonia. It has been investigated for the production of enantiomerically pure D-amino acids from the D,L-racemic amino acid amides that are readily derived from aldehydes, hydrogen cyanide, and ammonium.[1,13] Asano et al. reported that D-amino acids can be synthesized via full conversion from D,L-racemic amino acid amides using DaaA and L-α-amino-ε-caprolactam racemase, which catalyzes the racemization of all remaining L-amino acid amides.[11] These methods could synthesize all D-amino acids. However, unfortunately, only few DaaAs have been described;[14,15] thus, new D-amino acid amidases are still required in various applications.[1,13] However, the information of this enzyme was limited compared to L-amino acid amidases.

Recently, several highly sensitive genetic circuit-based biosensors have been developed for the high-throughput screening of novel and evolved enzymes and biological pathways for the synthesis of valuable biochemicals.[16−20] Nevertheless, amino acid amides are unsuitable for D-amino acid amidase screening of D-amino acid amidase because their enzymatic reaction products are rapidly metabolized. A BenR-based method for amino acid amidase screening uses benzamide as a substrate for cellular fluorescence.[21] However, this approach was inappropriate here as it responds to various enzymes in the presence of either D- or L-amino acids. A combination of substrate design and biosensor strategy was necessary to screen for D-amino acid amidohydrolase.

In this strategy, we previously showed that a phenyl moiety is a putative target in numerous enzyme substrates.[22] The phenyl moiety in artificial substrates has advantages such as

Received: September 23, 2021
Revised: December 23, 2021
Accepted: December 29, 2021
Published: January 7, 2022



https://doi.org/10.1021/acs.jafc.1c05834
J. Agric. Food Chem. 2022, 70, 1203−1211

intracellular stability; simple chemical synthesis; and a planar, compact, and rigid structure. Other structures of artificial substrates, except for phenyl moieties, can be designed for various functional groups considering the target enzyme activity. Here, we present a new strategy for screening D-amino acid amidases with an amidophenol substrate bearing an amide group as the target point of amidase activity. The dimethyl phenol regulatory protein (DmpR)-genetic enzyme screening system (GESS) can detect the released phenolic compounds as a transcription factor-based biosensor.[17] We applied this system to detect D-threonine amidases using D-threonine-2-hydroxyphenylamide (DTHPA) as an artificial substrate (Figure 1). This approach demonstrated the successful combination of a designed substrate with a genetic sensor to screen for microbial and engineered enzymes.

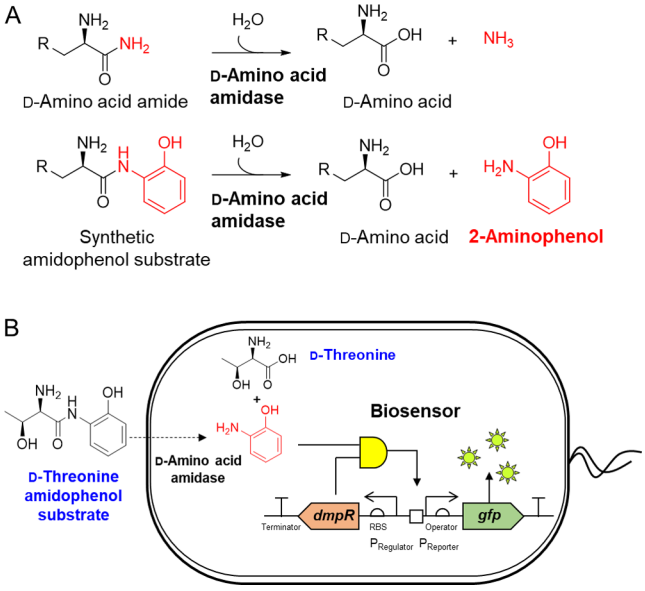


Figure 1. Development of a D-amino acid amidase-based screening method using a synthetic substrate and a genetic circuit. (A) D-amino acid amidase reaction with synthetic substrates. The amidase hydrolyzes the synthetic substrate in vivo and generates 2-aminophenol, which activates the genetic circuit. (B) D-amino acid amidase screening strategy. 2-Aminophenol from D-threonine phenylamide hydrolysis by D-amino acid amidase activates the reporter gene transcription.

MATERIALS AND METHODS

Materials. All chemical reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). DTHPA and D-threonine amide were synthesized by 4Chem Laboratory (Suwon, Gyeonggi-do, Korea). Restriction endonucleases, DNA cloning kits, pUC19, and *Escherichia coli* DH5α cells were purchased from New England Biolabs (Beverly, MA, USA). Plasmid DNA isolation and DNA extraction were performed with plasmid preparation kits (Promega, Madison, WI, USA). Oligonucleotide synthesis and sequencing were conducted by Macrogen (Daejeon, Korea).

Microorganisms, Plasmids, Media, and Culture Conditions. *Bacillus licheniformis* KCTC1659, *Bacillus amyloliquefaciens* KCTC13588, *Bacillus subtilis* KCTC2217, *Bacillus megaterium* KCTC3007, *E. coli* ER2566, and pET-28a (+) plasmid (Novagen, Darmstadt, Germany) were sources of D-amino acid amidase genomic DNA, host cells, and expression vectors, respectively. Recombinant *E. coli* cells for enzyme expression were cultivated in a 500 mL Lysogeny broth (LB) medium. When the bacterial OD_{600} = 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 0.1 mM final concentration to induce D-amino acid amidase expression and the culture was incubated at 16 °C and shaking at 150 rpm for 16 h.

DmpR(E135K)-GESS Chromosomal Version Construction. To detect D-amino acid amidases via fluorescence signals, a DmpR(E135K)-GESS genetic construct was integrated into the chromosome for energetic benefits such as reducing plasmids, proteins, and antibiotics. *E. coli* EPI300(DE3)[23] was selected as the parental strain to construct the D-aminopeptidase screening strain as the former could be further expanded with fosmid (or cosmid) metagenomes or T7 expression systems.

To construct the *E. coli* EPI300-(DE3Δ*bglA*::DmpRGESSE135K);mphGEP01(DE3) by chromosomal integration of the cassette containing the genetic construct, the *bglA* region from 300 bp upstream to 300 bp downstream (including the kanamycin resistance gene) was amplified by PCR from the KEIO collection strain BW25113Δ*bglA*::Km and subcloned into the pGEM T-easy vector following the manufacturer's protocol. The primers were bglA_300up and bglA_300dn. The pGESS (E135K) plasmid from a previous study[17] served as a chromosomal cassette template. The promoter was substituted for DmpR and the reporter protein was replaced with the HCE promoter (pHCE_F1 and pHCE_R1 primers) from pHCEIIB and sf-GFP (sfGFP_F1 and sfGFP_R1 primers), respectively.[24] The construct was ligated with a Gibson assembly kit (dmpR_F1, pGFP_F1, pGFP_R1, rrnBT_F1, tL3_R1, rrnBT_R1, rrnBT_int_F1, and tL3_int_F1 primers) into the cloned homologous region of the pGEM plasmid. The cassette was integrated by λ-red recombination into the chromosome using the pKD46 plasmid.[25] The kanamycin resistance gene in the chromosomal GESS strain was deleted by flippase recombination with pCP20.[26] All primer sequences are shown in Table S1.

Gene Cloning and Mutagenesis. D-stereospecific aminopeptidase from *Ochrobactrum anthropi* (OADaaA) was synthesized and subcloned into pET-28a (+) by Macrogen Co. (Seoul, Korea). The D-amino acid amidases from *B. licheniformis* (BLDaaA), *B. amyloliquefaciens* (BADaaA), *B. subtilis* (BSDaaA), and *B. megaterium* (BMDaaA) were amplified by PCR using genomic DNA isolated as templates. The fragments were amplified with the corresponding primers (Table 1) and subcloned by the Gibson assembly into pET-28a (+). The DNA was sequenced by Macrogen Co. (Daejeon, Korea). The amino acid sequences of the D-amino acid amidases were aligned with those of other amidases in GenomeNet ClustalW. A Diversify PCR random mutagenesis kit (Clontech Laboratories, Mountain View, CA, USA) was used for random library construction according to the manufacturer's protocol. There were two mutations

Table 1. Kinetic Parameters of Five F119 BMDaaA Variants for Various D-Amino Acid Amides

	D-Thr amide			D-Ala amide			D-Gln amide			D-Val amide			D-Met amide		
	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m
wild	1.659	0.001	0.0008	6.871	4.328	0.629	4.356	0.012	0.002	4.364	0.918	0.210	6.742	3.998	0.593
F119A	1.315	0.007	0.005	5.061	4.264	0.842	3.748	0.161	0.042	3.912	0.924	0.236	4.733	3.932	0.831
F119L	1.544	0.005	0.004	4.754	4.293	0.903	4.107	0.095	0.023	2.680	0.874	0.326	4.576	4.009	0.876
F119I	1.618	0.001	0.0007	6.98	2.66	0.381	4.395	0.073	0.016	6.599	0.319	0.048	6.429	2.141	0.333
F119V	1.676	0.001	0.0005	5.134	4.267	0.831	4.288	0.010	0.002	4.137	0.916	0.221	4.913	3.959	0.806

https://doi.org/10.1021/acs.jafc.1c05834
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per 1 kb and the library size was 5×10^5. Site-directed mutagenesis was performed using the primers synthesized by Macrogen (Seoul, Korea).

Fluorescence Analysis and Screening. The mphGEP01(DE3) cells were grown at 37 °C for 24 h in M9 medium (per liter: 12.8 g of $Na_2HPO_4 \cdot 7H_2O$, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 4 g of glucose, and 0.1 g of thiamine) supplemented with 50 μM 2-aminophenol. Fluorescent colonies were visualized under an epifluorescence microscope (AZ100-M; Nikon, Tokyo, Japan) fitted with a GFP filter (excitation: 455−485 nm; emission: 500−545 nm). For the liquid-phase mphGEP01 (DE3) fluorescence analysis, a single colony was inoculated in 2 mL of M9 medium. After cultivation at 37 °C for 16 h, 1% (v/v) seed was inoculated in 2 mL of fresh M9 media containing 0−50 μM 2-aminophenol. Fluorescence intensities were measured with FACS Aria (BD Biosciences, Franklin Lakes, NJ, USA) and a multilabel microplate reader (PerkinElmer, Billerica, MA, USA). To screen for new D-amino acid amidases in the liquid phase, 1% (v/v) mphGEP01 (DE3) seed culture and 1% (v/v) mixed bacteria were inoculated in 2 mL of M9 medium containing 200 μM DTHPA as a synthetic substrate. For the GESS fluorescence analysis of various liquid-phase D-amino acid amidases, a single mphGEP01(DE3) colony harboring OADaaA, BADaaA, BMDaaA, BSDaaA, and BLDaaA in pET28a (+) was inoculated in 2 mL of M9 medium supplemented with 25 μg mL^{-1} kanamycin. After cultivation at 37 °C for 16 h, 1% (v/v) culture was inoculated in 2 mL of fresh M9 medium containing kanamycin plus 0.1 mM IPTG and 200 μM DTHPA. Fluorescence intensity was measured after 6 h at 37 °C in a multilabel microplate reader at excitation and emission wavelengths of 485 nm and 535 nm, respectively. For the mutant library screening, cells with a high fluorescence intensity were sorted in a FACSAriaIII instrument (BD Biosciences, Franklin Lakes, NJ, USA), and the data were analyzed with FlowJo (Tree Star, Ashland, OR, USA). Time-lapse fluorescence intensity was analyzed in a plate reader (Infinite M200 Pro, Tecan, Männedorf, Switzerland).

D-Amino Acid Amidase Purification. *E. coli* C2566 cells harboring pET28a-D-amino acid amidases were grown in a LB medium at 37 °C until OD_{600} = 0.4 − 0.5. The cells were induced with 0.1 mM IPTG and the culture was transferred to a fresh medium at 20 °C and incubated for 18 h. The cells were harvested by centrifugation at 2000*g* for 10 min and resuspended in a lysis buffer (300 mM KCl, 50 mM KH_2PO_4, and 5 mM imidazole [pH 8.0]). Cell extracts were prepared by sonication, purified with Profinia (Bio-Rad Laboratories, Hercules, CA, USA), and mixed with a reaction buffer containing 50 mM *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) buffer (pH 9.5) and 1 mM various D-amino acid amides at 55 °C for 10 min. Protein was quantified by the Bradford method. Purified proteins were confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Effects of Metal Ions, pH, and Temperature. The reaction was run for 10 min at 55 °C in 50 mM CHES buffer (pH 9.5) containing 1 mM D-threonine amide, 0.5 U mL^{-1} enzyme, and 0.5 mM Co^{2+}. The effect of metal ions on BMDaaA was assessed by adding 0.5 mM Mn^{2+}, Zn^{2+}, Co^{2+}, or Mg^{2+} to the enzyme. The effects of Co^{2+} were investigated using a concentration range of 0−5 mM. The effects of pH on BMDaaA activity were evaluated by varying the former between 6.5 and 10 with 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (pH 6.5−7.5), 50 mM EPPS buffer (pH 7.5−8.5), and CHES buffer (pH 8.5−10.0). The effects of temperature on enzyme activity were measured by modulating the former between 35 and 75 °C. One unit (U) D-amino acid amidase activity was defined as the amount of enzyme required to produce 1 μmol D-threonine per minute at 55 °C and pH 9.5.

Substrate Specificity and Kinetic Parameters. To measure the D-amino acid amide specificity of wild-type and F119 variant BMDaaA, enzyme reactions were conducted at 55 °C in 50 mM CHES buffer (pH 9.5) containing 2.5 mM D-alanine amide, D-methionine amide, D-valine amide, D-glutamine amide, D-threonine amide, L-alanine amide, L-methionine amide, L-valine amide, L-glutamine amide, and L-threonine amide. The enzyme concentration was adjusted in the range of 0.1−25 U mL^{-1} and the reaction time was 5 min. The reaction was stopped by boiling at 100 °C for 15 min. The specific activity was defined as the increase in D-amino acid produced per unit enzyme quantity per unit reaction time. One unit enzyme activity was defined as the amount required to produce 1 μmol D-amino acid per minute at 55 °C and pH 9.5. The kinetic parameters for the wild-type and the F119A, F119L, F119I, and F119V variants of BMDaaA were determined by running the reactions for 10−15 min at 55 °C in 50 mM CHES buffer (pH 9.5) and varying the amounts of D-alanine amide, D-methionine amide, D-valine amide, D-glutamine amide, and D-threonine amide in the range of 0.5−1000 μM. K_m (mM) and k_{cat} (min^{-1}) were determined by nonlinear regression in GraphPad (GraphPad Software, La Jolla, CA, USA).

Bioconversion and Recycle Reaction. The time courses of the conversion of D-amino acid amide into D-amino acid by wild-type and F119A variant BMDaaA were determined at 55 °C for 180−240 min using 50 mM CHES buffer (pH 9.5) containing 0.005−0.3 mg mL^{-1} purified enzyme, 10 mM D-amino acid amide including D-threonine amide and D-alanine amide, and 0.5 mM Co^{2+}. Samples were withdrawn at regular time intervals and assayed. To investigate enzyme activity recycling for D-threonine production, 100 μL of 0.5 mg mL^{-1} purified enzyme was immobilized by gentle mixing with 100 μL of nickel-nitrilotriacetic acid (Ni-NTA) resin (Novagen, Darmstadt, Germany) at 4 °C for 30 min and washing twice in phosphate-buffered saline (PBS). The recycling reactions were analyzed at 55 °C for 3 h using 50 mM CHES buffer (pH 9.5) containing 10 mM D-threonine amide, 0.5 mg mL^{-1} immobilized enzymes, and 0.5 mM Co^{2+}. After the reactions, supernatants were withdrawn and washed thrice with PBS. Then, 50 mM CHES buffer (pH 9.5) containing 10 mM D-threonine amide was added to the washed, immobilized enzymes and allowed to react for 3 h. The foregoing reaction was repeated 5×.

Analytical Methods. The D-amino acid and D-amino acid amide concentrations in the reaction mixture were determined by HPLC (UV 340 nm; Agilent Technologies, Santa Clara, CA, USA) fitted with an Eclipse XDB-C18 column (4.6 mm × 150 mm). The mobile phase consisted of 30:70 v/v methanol:50 mM sodium acetate (pH 5.9; 3 min) and a 30−70% methanol gradient (6 min) at 25 °C after derivatization with *o*-phthalaldehyde (OPA) plus *N*-acetylcysteine (NAC). The OPA/NAC derivatization reagent was prepared by dissolving 8 mg of OPA and 10 mg of NAC in 1 mL of methanol. Each 250 μL vial holding the derivatization reaction mixture contained 25 μL of amino acid sample, 50 μL of OPA/NAC reagent, and 175 μL of 0.4 M sodium borate buffer (pH 10.4).

Homology Modeling. The BMDaaA homology model was plotted in Modeller v. 9.20 (https://salilab.org/modeller) and used the *B. subtilis* (PDB ID: 1HI9) D-aminopeptidase crystal structure as the template.[27] The structure with the lowest discrete optimized protein energy (DOPE) score was selected and used in the subsequent analyses. The DTHPA ligand was docked onto the BMDaaA model structure with Autodock Vina v. 1.1.2 (http://vina.scripps.edu).[28] The Zn charges on the BMDaaA model structure were manually set to +2e for ligand docking purposes.

RESULTS AND DISCUSSION

Detection of D-Amino Acid Amidase. DaaA successfully catalyzes the hydrolysis of the designed substrate, and 2-aminophenol may be released as a product. Here, we tested the 2-aminophenol detection by DmpR-GESS via fluorescence reporter expression (Figure 1B). The designed amidophenol could indicate DaaA-related activity in native bacteria and directed evolution libraries. We used a mutant DmpR(E135K)-GESS to enhance 2-aminophenol detection sensitivity.[17] A single copy of the DmpR(E135K)-GESS was integrated into the *E. coli* EPI300 [mphGEP01(DE3)] chromosome to facilitate screening and attenuate background noise. The sensor cell was grown in M9 minimal medium and used to test the fluorescence response to 2-aminophenol. The growth

https://doi.org/10.1021/acs.jafc.1c05834
J. Agric. Food Chem. 2022, 70, 1203−1211

medium enhanced DmpR sensitivity via a sigma 54-related stringent response.[29,30] Chromosomal integration of the genetic circuit attenuated background noise while increasing sensitivity toward 2-aminophenol (Figure 2A). The sensor cell

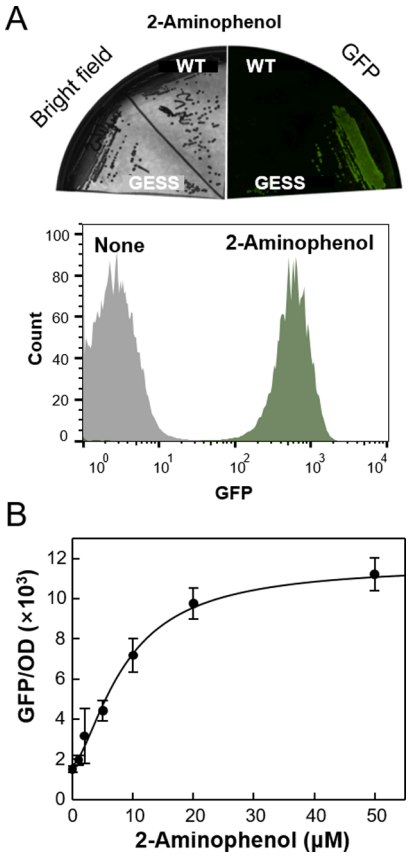

Figure 2. Biosensor cell fluorescence response to 2-aminophenol. (A) Genetic circuit-based biosensor fluorescence analysis. The genetic circuit is integrated into the host genome. Colony and single-cell fluorescence intensities were determined in the M9 medium (per liter: 12.8 g of $Na_2HPO_4{\cdot}7H_2O$, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 4 g of glucose, and 0.1 g of thiamine) supplemented with 50 μM 2-aminophenol using fluorescence microscopy and flow cytometry, respectively. (B) Quantitative fluorescence analysis of the genetic circuit with various 2-aminophenol concentrations in the M9 medium. Error bars represent the standard deviation of triplicate experiments.

cytometry response was consistent with the colony images. The DmpR-GESS response level was analyzed using mphGEP01 (DE3) cells fluorescence signals in M9 medium containing 0−50 μM 2-aminophenol (Figure 2B). The mphGEP01 (DE3) detected 1 μM 2-aminophenol and could, therefore, screen very low enzyme activity. The designed substrate and phenol sensor system were used to screen D-amino acid amidases. DaaA from *O. anthropi* (OADaaA) served as a control.[13,14] The mphGEP01 (DE3) cells harboring OADAP in pET28a (+) strongly fluoresced after 6 h in the presence of the artificial substrate (Figure 3). In contrast, the sensor cells harboring empty pET28a (+) (negative control) displayed no measurable fluorescence signal (Figure S1). The results confirmed that the artificial substrate penetrated into the cells and the DmpR-GESS cells detected the 2-aminophenol hydrolyzed by the cytosolic expressed OADaaA. We then performed a time-lapse study of mphGEP01 (DE3) cells

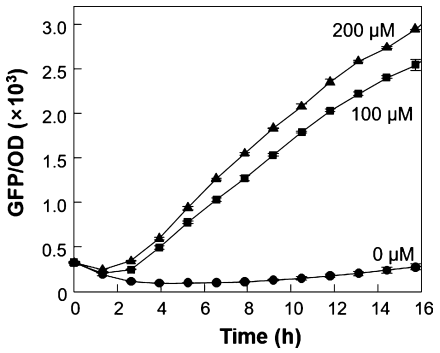

Figure 3. D-Amino acid amidase activity detection with the DmpR-GESS system. Time-lapse fluorescence profiles of sensor cells harboring pET-28a plasmids encoding D-aminopeptidase from *O. anthropi* (OADaaA) at 0 μM (closed circle), 100 μM (closed square), and 200 μM (closed triangle) D-threonine-2-hydroxyphenylamide in M9 medium. Values represent the means ± SDs of triplicates.

harboring OADaaA-pET28a (+) or empty pET28a (+) as a negative control construct in the presence of 0, 100, or 200 μM artificial substrate. In this assay, we measured green fluorescent protein (GFP) fluorescence. In the presence of 100 μM DTHPA, the cells harboring OADaaA-pET28a (+) fluoresced within 3 h and the intensity had increased by ∼10 fold after 6 h (Figure 3). After 8 h, slight fluorescence was detected in the empty-vector control cells in the presence of the artificial substrate (data not shown). To minimize false positives, we selected 6 h as the appropriate reaction time to analyze D-amino acid amidase activity on the artificial substrate.

Measurement of D-Amino Acid Amidase Activity Using DmpR-GESS. As we confirmed the detection of D-amino acid amidase activity using DmpR-GESS, we tested whether our assay strategy could detect D-amino acid amidase activity in other microbes. To screen for D-amino acid amidase activity, co-cultures of 14 candidate microbes and sensor cells were prepared. Each strain was separately cultivated in LB medium and washed with M9 medium. Each 5% (v/v) culture solution comprising mphGEP01 (DE3) cells and individual microbes was mixed with 200 μM DTHPA and incubated at 30 °C for 6 h. Several *Bacillus* species fluoresced more strongly than others (Figure 4A). As the genetic circuit has micromolar sensitivity to the designed product, it can detect even slight enzymatic activity in native strains.

Based on known *Bacillus* genomic sequences, we speculated on the genes responsible for 2-aminophenol release from the artificial substrate that is the binuclear Zn^{2+}-dependent D-aminopeptidase from *B. subtilis*.[31] Based on this information, we identified sequences of putative D-aminopeptidase as homologous enzymes compared to Zn^{2+}-dependent D-aminopeptidase from *B. subtilis*, and then, we cloned four D-aminopeptidases from *B. licheniformis*, *B. amyloliquefaciens*, *B. subtilis*, and *B. megaterium* into pET28a (+), transformed the enzyme-bearing plasmids into sensor cells, and cultivated them for 6 h in the presence or absence of the artificial substrate. All enzymes showed D-amino acid amidase activity toward the artificial substrate and strongly fluoresced (Figure. S2). The D-aminopeptidases from *B. licheniformis*, *B. amyloliquefaciens*, *B. subtilis*, and *B. megaterium* were purified by His-tag affinity chromatography and tested positive for D-amino acid amidase activity toward D-threonine amide (Figure 4B). Although the actual activity of the selected recombinant D-aminopeptidase was confirmed in HPLC analysis, there was no close

https://doi.org/10.1021/acs.jafc.1c05834
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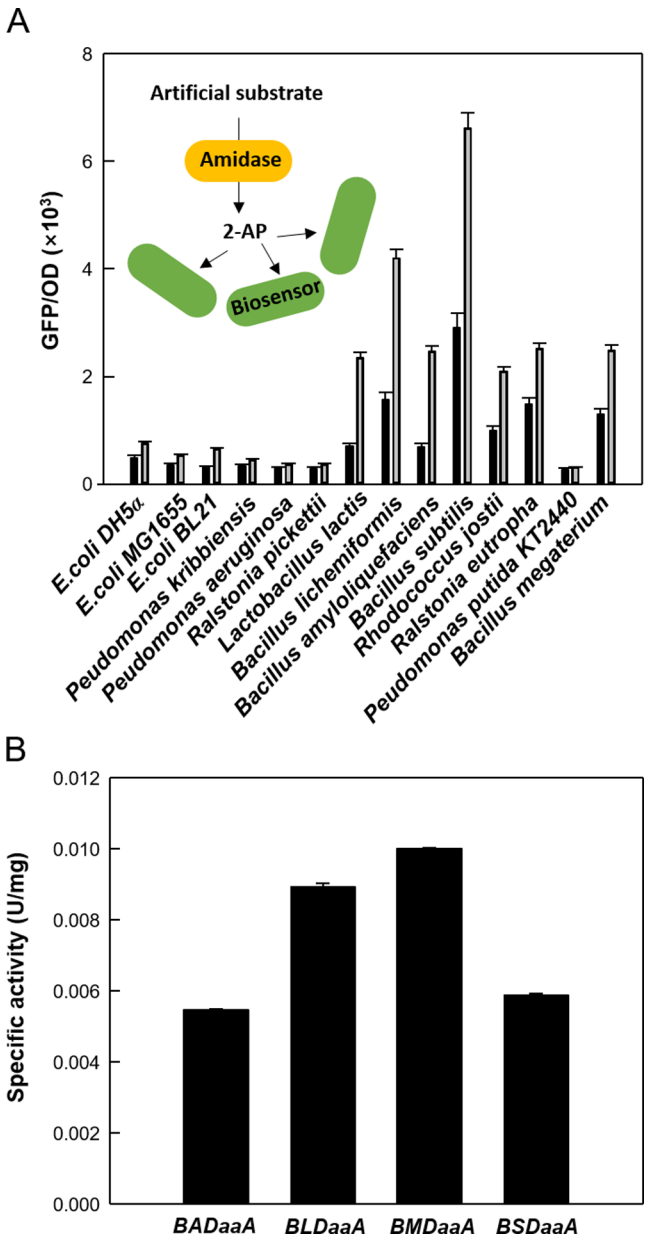

Figure 4. Fluorescence-based investigation of various microbes for D-amino acid amidase activity. (A) Identification of amidase in microbes using biosensor cells. Culture broth as M9 medium of the listed microbes were reacted with the sensor cells for D-amino acid amidase activity. Fluorescence intensity of wild-type plus DmpR-GESS cells in the absence of substrate (black bars) or in the presence of 200 μM D-threonine 2-hydroxyphenylamide (gray bars) at 5 h. Values represent the means ± SDs of triplicate measurements. (B) HPLC of D-amino acid amidase activity in *Bacillus* strains. D-Threonine amide was degraded by D-amino acid amidase to D-threonine and ammonia. Purified enzyme was combined at 55 °C for 10 min with 50 mM CHES buffer (pH 9.5) containing 1 mM D-threonine amide. Values represent the means ± SDs of triplicates.

correlation between the fluorescence intensity and enzyme activity. This may be because of the different expression levels of enzymes in microbes and different cell growth during co-cultivation. HPLC disclosed comparatively higher amidase activity in *B. megaterium*, whereas the enzyme expression levels did not significantly differ among cells (Figure S3). Hence, D-aminopeptidases from *B. megaterium* (BMDaaA) were selected for subsequent D-amino acid amidase activity characterization and engineering.

Directed Evolution of D-Amino Acid Amidases. The artificial substrate and the phenol sensor system then constituted a random mutagenesis template to engineer D-amino acid amidase. A sensor cell harboring the mutant library was cultivated at 30 °C for 3 h in M9 medium supplemented with 0.1 mM IPTG and 200 μM DTHPA. Cells expressing high fluorescence intensity (top 1%) were collected by flow cytometry (Figure 5A). Individual colonies were obtained after

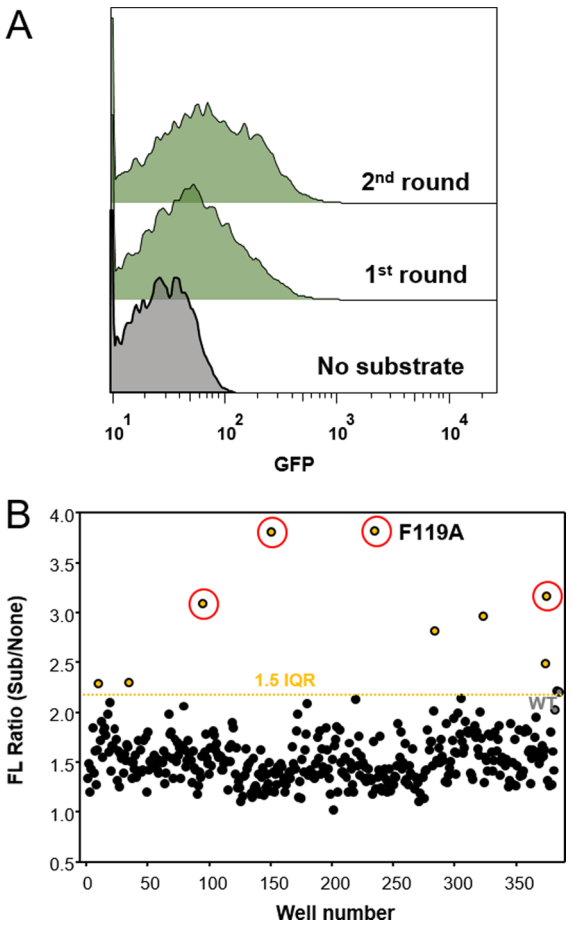

Figure 5. mBMDaaA engineering via genetic circuit and synthetic substrate. (A) Mutant library fluorescence histograms plotted by flow cytometry. Cells were cultivated in the M9 medium containing 200 μM D-threonine-2-hydroxyphenylamide. Strongly fluorescent cells were sorted by flow cytometry. (B) In cell assay of hits from flow cytometry. Candidates with fluorescence intensity >1.5× the inter-quartile range (IQR) were selected for further sequence analysis.

two enrichment rounds. Well plate cultivation was conducted to identify beneficial mutations formed by random mutagenesis (Figure 5B). A sequencing analysis detected the F119A mutant in four candidates among nine hits. Hence, F119A was subjected to further testing (Table S2).

Characterization of Evolved D-Threonine Amidase. Wild-type BMDaaA was purified by His-tag affinity chromatography and characterized as a 31.2 kDa protein (Figure S4A) with D-amino acid amidase activity. Maximum enzyme activity toward 1 mM D-threonine amide was detected at 55 °C and pH 9.5 (Figures S4B,C). A previously reported D-aminopeptidase from *B. subtilis* was a binuclear Zn^{2+}-dependent

https://doi.org/10.1021/acs.jafc.1c05834
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enzyme.[32] Thus, BMDaaA was assayed at 55 °C and pH 9.5 with 1 mM D-threonine amide in the presence or absence of 1 mM of various metal ions after treatment with 10 mM EDTA (Figure S5A). Co^{2+}, Mg^{2+}, Zn^{2+}, and Mn^{2+} enhanced BMDaaA activity relative to the control. Co^{2+} was the most effective and its optimal concentration was 0.5 mM (Figure S5B). In the subsequent assays, then, D-amino acid amide deamination by BMDaaA was performed in the presence of 0.5 mM Co^{2+}. Based on the optimal BMDaaA conditions, we investigated the substrate specificity of wild-type and F119 variant BMDaaA. We subjected them to D-alanine amide, D-methionine amide, D-valine amide, D-glutamine amide, D-threonine amide, L-alanine amide, L-methionine amide, L-valine amide, L-glutamine amide, and L-threonine amide. BMDaaAs had the highest specific activity toward D-alanine amide (21.07 ± 0.21 U mg^{-1}) followed by D-methionine amide, D-valine amide, D-glutamine amide, and D-threonine amide (Table S3). However, it exhibited no activity toward L-alanine amide, L-methionine amide, L-valine amide, L-glutamine amide, or L-threonine amide (data not shown). Therefore, the enzyme isolated here has D-stereospecific amino acid amidase activity.

The catalytic efficiency (k_{cat}/K_m) of the F119A variant for D-threonine amide was 6.2-fold higher than that of the wild-type (Table 1). Moreover, the F119A variant had ∼21 fold stronger activity on D-glutamine amide than wild-type BMDaaA. The catalytic efficiency of wild-type BMDaaA on various substrates was highest for D-alanine amide (0.629 min^{-1} mM^{-1}). K_m for the F119A, F119L, and F119V variants did not markedly differ from those of wild-type BMDaaA. Consequently, the strategy to design new substrate for evolutionary engineering of enzymes increased the catalytic turnover while maintaining the substrate binding similarity between the artificial and natural substrates.

Structural Consequence Analysis of BMDaaA. The *B. subtilis* D-amino acid amidase crystal structure was a decamer stacking a barrel-shaped complex by two ring-shaped pentamers.[32] The active sites are located in the inner cavity with a 20 Å channel to compartmentalize the protease activity. A sequence alignment of 45 D-aminopeptidases from the NCBI database disclosed that all metal-related residues in D-amino acid amidases are perfectly conserved (Figure S6). However, Phe^{119} screened by random mutagenesis at the active site varied among D-amino acid amidase family members. A ligand-docking study showed that Phe^{119} is located near the D-threonine R-group (Figure 6). A single-point mutation study revealed that Phe^{119} may play an important role in the hydrolysis of various D-amino acid amides with different R-groups. A molecular docking simulation analysis showed that the cavity between Ser^{61}, Met^{63}, and Met^{117} is large enough to accommodate a bulky functional group in the carboxylic region. Thus, the phenol moiety in the artificial substrate could be recruited into the cavity (Figure 6). Further mutational studies and X-ray analyses must be conducted to elucidate BMDaaA D-amino acid amidase activity. The substrates designed here should be permeable to the biosensor cells in the absence of any interference from intracellular factors. Living cells are highly permeable to the phenol moiety of the synthetic substrate and do not degrade it.[33] Though D-threonine amidase is highly D-stereospecific, it could be screened and rapidly engineered for elevated enzyme activity via the biosensor used and the substrate designed in the present study.

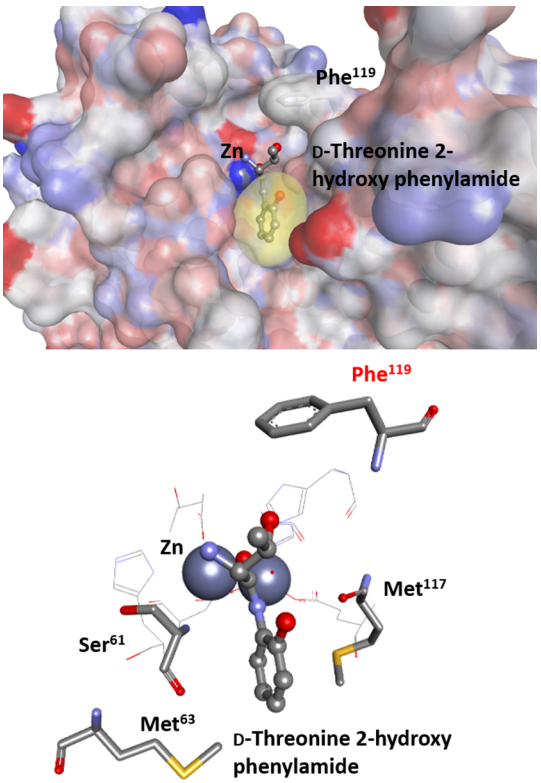

Figure 6. BMDaaA structural consequence analysis. D-threonine 2-hydroxyphenylamide and Zn^{2+} in the BMDaaA active site are represented by a ball-stick and CPK, respectively. The phenol moiety is represented by the yellow area. Ser^{61}, Met^{63}, Met^{117}, and Phe^{119} in the BMDaaA structure are represented by sticks.

D-Threonine Bioconversion Using BMDaaA and F119A Variant. The F119A and wild-type enzyme bioconversion profiles were investigated using D-threonine amide and D-alanine amides as substrates. Each enzyme (0.3 mg mL^{-1}) was incubated with 10 mM D-threonine amide. F119A displayed 95% molar conversion, whereas the wild-type showed ∼70% molar conversion yield after 120 min (Figure 7A). When D-alanine amide was used as the substrate for 0.005 mg mL^{-1} of each enzyme, both F119A and the wild-type presented with molar conversion profiles resembling those for D-threonine (Figure 7B). The wild-type converted only 88% D-alanine in 240 min, while the F119A variant converted 9.7 mM D-alanine from 10 mM D-alanine amide in 180 min and the molar conversion was 97%. We then tested the enzymatic bioconversion of D-threonine by examining recycling reactions with His-tag immobilization of the F119A variant and wild-type BMDaaA. The F119A variant and wild-type BMDaaA immobilized on Ni-NTA with His-tag demonstrated 85% and 72% molar conversion, respectively, after 3 h. After five repetitions of the recycling reaction, the molar conversion of D-threonine from D-threonine amide by wild-type BMDaaA decreased from 72 to 22%. In contrast, while the F119A variant provided an initial 85% molar conversion of D-threonine from D-threonine amide, it nonetheless maintained 70% molar conversion (1.2 fold reduction) even after five cycles (Figure 8). As both enzymes revealed similar residual activity after 36 h, the observed discrepancy may be explained by differences in their catalytic efficiencies (Figure S7).

Here, we designed and empirically tested an amidohydrolase screening strategy based on an amidophenol substrate and a

https://doi.org/10.1021/acs.jafc.1c05834
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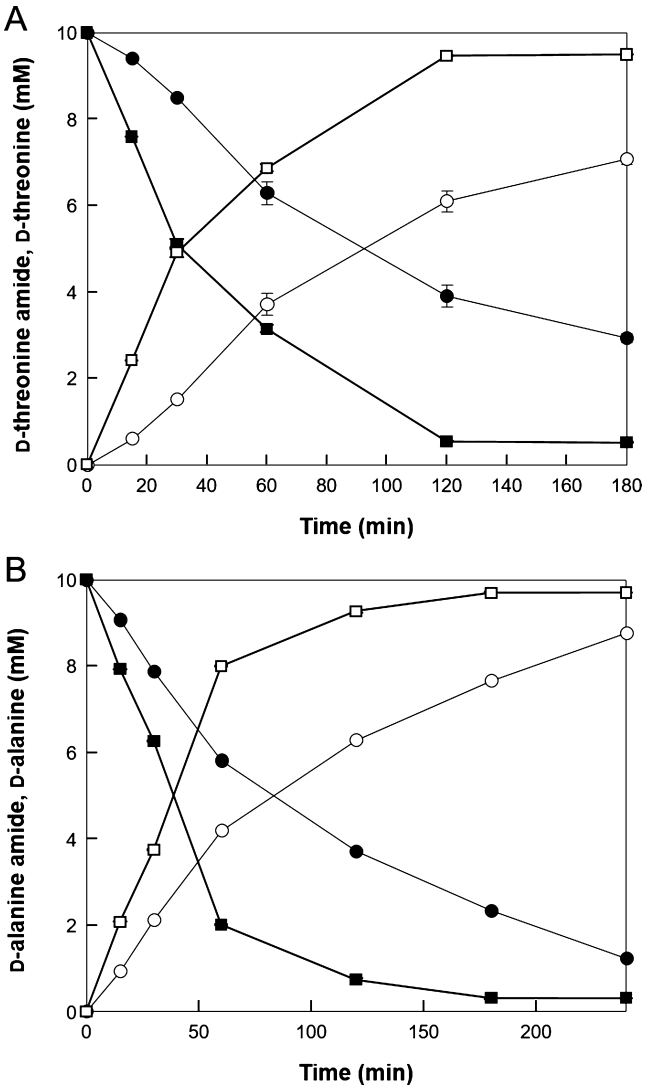

Figure 7. Time course of D-amino acid production from D-amino acid amide by wild and F119A variant BMDaaA. (A) Conversion of D-threonine (Open circle: wild, open square: F119A variant) from 10 mM D-threonine amide (Closed circle: wild, closed square: F119A variant). The reactions were performed in 50 mM CHES buffer (pH 9.5) containing 10 mM D-threonine amide, 0.3 mg mL^{-1} purified enzyme, and 0.5 mM Co^{2+} at 55 °C. (B) Conversion of D-alanine (Open circle: wild, open square: F119A variant) from 10 mM D-alanine amide (Closed circle: wild, closed square: F119A variant). The reactions were performed in 50 mM CHES buffer (pH 9.5) containing 10 mM D-alanine amide, 0.005 mg mL^{-1} purified enzyme, and 0.5 mM Co^{2+} at 55 °C. Data are presented as the mean ± standard error of the mean (SEM) of three separate experiments.

transcriptional regulator-based genetic circuit. This configuration could effectively screen enzymes in combination with a substrate containing an amide bond for the release of 2-aminophenol in response to enzymatic reactions such as those catalyzed by BMDaaA. We identified and engineered a D-stereospecific amino acid amidase with comparatively high bioconversion and recycling reaction efficiencies. Further engineering of DmpR can be performed to increase the substrate specificity of 2-aminophenol. The method developed herein could be expanded to produce other D-amino acid amidases for the generation of various D-amino acids.

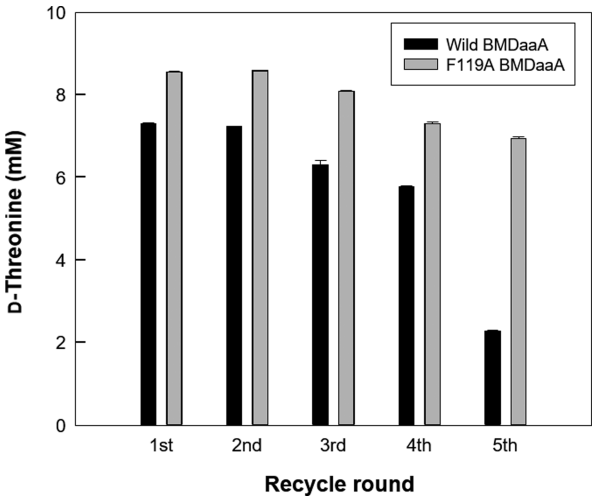

Figure 8. Recycle reaction of D-threonine production from 10 mM D-threonine amide using immobilized wild-type (black bar) and F119A variant (gray bar) BMDaaA. Data are mean ± standard error of the mean of three separate experiments. Purified enzymes (0.5 mg mL^{-1}) were immobilized on 100 μL of Ni-NTA resin. Reactions were performed in 50 mM CHES buffer (pH 9.5) containing 10 mM D-threonine amide, 0.5 mg mL^{-1} immobilized enzyme, and 0.5 mM Co^{2+} at 55 °C for 3 h and repeated five times.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c05834.

List of primers used in the study; identification of mutations in mBMDaaA hits from DmpR-GESS screening; comparison of specific activity of F119 variant BMDaaA toward various D-amino acid amides ; fluorescence intensity of sensor cells harboring pET-28a plasmid or pET-28a plasmid encoding D-aminopeptidase from *O.chrobactrum anthropi* (OADaaA); fluorescence analysis of D-amino acid amidase from *Bacillus* strains using DmpR-GESS; expression level of D-amino acid amidase from various *Bacillus* strains; expression and effects of temperature and pH for the activity of purified D-amino acid amidase of *B. megaterium*; effects of various metal ions for the activity of D-amino acid amidase from *B. megaterium*; sequence alignment of metal-associated residues and Phe^{119} in 45 D-aminopeptidases; and time-lapse profile of residual activity of wild-type and F119A mutant D-amino acid amidase from *B. megaterium* The authors state that there are no conflicts of interest to declare (PDF)

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https://doi.org/10.1021/acs.jafc.1c05834
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Funding

This work was supported by the Bio & Medical Technology Development Program (grant number: 2021M3A9I4022731) of the National Research Foundation funded by the Ministry of Science and ICT of the Republic of Korea, the C1 Gas Refinery Program of the Ministry of Science and ICT (No. 2018M3D3A1A01056181), the National Research Council of Science & Technology (NST) grant by the Korea government (MSIT) (No. CAP20022-000), and the Korea Research Institute of Bioscience and the Biotechnology (KRIBB) Research Initiative Program (KGM5402221).

Notes

The authors declare no competing financial interest.

ABBREVIATION AND NOMENCLATURE

DaaA, D-amino acid amidase; GESS, genetic enzyme screening system; DTHPA, D-threonine-2-hydroxyphenylamide; GFP, green fluorescence protein; OADaaA, D-amino acid amidase from *O. anthropi*; BLDaaA, D-amino acid amidase from *B. licheniformis*; BADaaA, D-amino acid amidase from *B. amyloliquefaciens*; BSDaaA, D-amino acid amidase from *B. subtilis*; BMDaaA, D-amino acid amidase from *B. megaterium*; LB, lysogeny broth; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; mphGEP01(DE3), *E. coli* EPI300(DE3) with DmpR(E135K)-GESS integrated into the chromosome

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