Dipeptide synthesis by internal adenylation domains of a multidomain enzyme involved in nonribosomal peptide synthesis

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The adenylation domain of nonribosomal peptide synthetase (NRPS) is responsible for its selective substrate recognition and activation of the substrate (yielding an acyl-O-AMP intermediate) on ATP consumption. DhbF is an NRPS involved in bacillibactin synthesis and consists of multiple domains [adenylation domain, condensation domain, peptidyl carrier protein (PCP) domain, and thioesterase domain]; DhbFA1 and DhbFA2 (here named) are “internal” adenylation domains in the multidomain enzyme DhbF. We firstly succeeded in expressing and purifying the “internal” adenylation domains DhbFA1 and DhbFA2 separately. Furthermore, we initially demonstrated dipeptide synthesis by “internal” adenylation domains. When glycine and L-cysteine were used as substrates of DhbFA1, the formation of N-glycyl-L-cysteine (Gly-Cys) was observed. Furthermore, when L-threonine and L-cysteine were used as substrates of DhbFA2, N-L-threonyl-L-cysteine (Thr-Cys) was formed. These findings showed that both adenylation domains produced dipeptides by forming a carbon-nitrogen bond comprising the carboxyl group of an amino acid and the amino group of L-cysteine, although these adenylation domains are acid-thiol ligase using 4¢-phosphopantetheine (bound to the PCP domain) as a substrate. Furthermore, DhbFA1 and DhbFA2 synthesized oligopeptides as well as dipeptides.

Key Words: adenylation domain; nonribosomal peptide synthetase; peptide bond; superfamily of adenylate-forming enzymes

Abbreviations: NRPS, nonribosomal peptide synthetase; PCP domain, peptidyl carrier protein domain; DHB, 2,3-dihydroxybenzoic acid; Gly-Cys, N-glycyl-L-cysteine; Thr-Cys, N-L-threonyl-L-cysteine; Ser-Cys, L-seryl-L-cysteine; Cys-Gly, L-cysteinyl-glycine; PPI, pyrophosphate

Introduction

Bacillibactin (2,3-dihydroxybenzoate-glycine-L-threonine trimeric ester) (Fig. 1) is a siderophore produced by bacteria under iron limitation to scavenge ferric ions (Abergel et al., 2009; Barry and Challis, 2009; Dertz et al., 2006). It is synthesized by nonribosomal peptide synthetases (NRPSs) (Finking and Marahiel, 2004) which are often involved in the synthesis of numerous secondary metabolites, mainly small peptides. Some of these peptides are antimicrobial, antiviral, immunosuppressant, and cytostatic agents, which have great pharmaceutical potential (Labby et al., 2015; Nguyen et al., 2006; Roongsawang et al., 2011). NRPSs are multidomain enzymes catalyzing the assembly of peptides through a thioester-templated mechanism. A general NRPS system consists of sets of protein modules that function as an assembly line for amino acid selection, activation, loading,
and coupling (Hur et al., 2012; Tanovic et al., 2008). Initially, an adenylation domain produces an aminoacyl-O-AMP and then covalently tethers it to the terminal thiol of the 4’-phosphopantetheine arm of a peptidyl carrier protein (PCP) domain (also known as a thiolation domain). A condensation domain forms a peptide/amide bond during peptide chain elongation. Finally, a thioesterase domain releases the full-length product from the assembly line (Trauger et al., 2000).

For bacillibactin biosynthesis (May et al., 2001), the following model was proposed. 2,3-Dihydroxybenzoic acid (DHB) is firstly activated by the stand-alone adenylation domain (DhbE) (May et al., 2002), and subsequently the activated DHB is transferred to the PCP domain of DhbB to yield DHB-S-PCP/DhbB. DHB is covalently attached to the 4’-phosphopantetheine thiol of PCP/DhbB via a thioester bond; DHB-S-PCP/DhbB is formed (Fig. 2A). On the other hand, glycine binds to the corresponding PCP domain in DhbF through the action of one internal adenylation domain of DhbF, Gly-S-PCP1/DhbF being formed (Fig. 2B, left). Thr-S-PCP2/DhbF is also formed by the other internal adenylation domain of DhbF using l-threonine (Fig. 2D, left). We named these internal DhbF adenylation domains DhbFA1 and DhbFA2, respectively. DHB-Gly-S-PCP1/DhbF (Fig. 2C) and DHB-Gly-Thr-S-PCP2/DhbF (Fig. 2E) are in turn produced through the catalytic control of the condensation domain of DhbF. And DHB-Gly-Thr binds to the thioesterase domain of DhbF (TE/DhbF) through ester bond formation at the side chain hydroxyl group of the active site serine residue of TE/DhbF, DHB-Gly-Thr-O-TE/DhbF being formed. Subsequently, a second round of DHB-Gly-Thr unit is synthesized and binds to the PCP domain of DhbF, DHB-Gly-Thr-S-PCP2/DhbF being formed. (DHB-Gly-Thr)_2-O-TE/DhbF was formed through binding of DHB-Gly-Thr-O-TE/DhbF and DHB-Gly-Thr-S-PCP2/DhbF. By repetition of this reaction, linear (DHB-Gly-Thr)_n-O-TE/DhbF is produced, and it is then released through cyclization as a trilactone (DHB-Gly-Thr)_n. The cyclization is catalyzed by the thioesterase domain of DhbF (May et al., 2001; Trauger et al., 2000).

We previously reported that an acyl-CoA synthetase (AcsA) involved in the nitrile-degradative pathway (Kobayashi and Shimizu, 1998) surprisingly synthesized an N-acyl compound when a suitable acid and l-cysteine were used as substrates (Abe et al., 2008). More recently, we also found the “stand-alone” adenylation domain, DhbE, of Bacillus subtilis catalyzed a novel N-acylation reaction, when an aromatic acid and l-cysteine were used as substrates (Abe et al., 2017). While the previously reported DhbE is a “stand-alone” adenylation domain, we here examined the N-acylation enzyme activity of “internal” adenylation domains, DhbFA1 and DhbFA2.

**Materials and Methods**

**Materials.** L-Cysteine was purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO). ATP and AMP were purchased from Kishida Chemical Co., Ltd. (Osaka, Japan). Amino acids (glycine, l-alanine, l-valine, l-leucine, l-isoleucine, l-serine, l-threonine, l-methionine, l-aspartic acid, l-asparagine, l-glutamic acid, l-glutamine, l-arginine, l-lysine, l-histidine, l-phenylalanine, l-tyrosine, l-tryptophan, l-proline, d-alanine, d-valine, d-leucine, d-serine, d-threonine, d-methionine, d-aspartic acid, d-asparagine, d-arginine, d-lysine, d-histidine, d-phenylalanine, d-tyrosine, d-tryptophan, and d-proline) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and...
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Nacalai Tesque (Kyoto, Japan). Dipeptides (Cys-Gly, Gly-Gly, Gly-Thr, Ala-Gly, Ala-Thr, and Thr-Gly) were purchased from Peptide Institute, Inc. (Osaka, Japan). The pentapeptide (Cys-Gly-Gly-Arg-Glu) and decapeptide (Cys-Gly-Gly-Arg-Glu-Ser-Gly-Ser-Gly-Ser) were prepared by Operon Biotechnology Inc. (Tokyo, Japan). Other reagents were purchased from Wako Pure Chemical Industries, Ltd.

Expression and preparation of internal adenylation domain proteins of DhbF. The programs Clustal Omega was used for creating multiple sequence alignments. Based on a multiple sequence alignment of enzymes belonging to the superfamily of adenylate-forming enzymes (Conti et al, 1996; Ehmann et al., 2000; Gulick, 2009) (Fig. 3), we determined the structural genes of internal adenylation domains (designated as dhbFA1 or dhbFA2) of DhbF (NCBI accession number AAD56240). DhbFA1 (amino acids 436–969 of DhbF) and DhbFA2 (amino acids 1492–2044 of DhbF) (Fig. 3) were used in this study.

(i) dhbFA1 The DNA fragment of dhbFA1 was amplified by PCR using chromosomal DNA of Bacillus subtilis strain 168 as a template. The following pair of oligonucleotide primers was used: 5'-AAAACATATGCTGG-GCCAGAGGAGAAAGAA-3' (NdeI recognition site is underlined) and 5'-TTTCTTCGACTATCTTCCTCGAGGTC-3' (XhoI recognition site is underlined). The
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PCR product was digested with NdeI and XhoI, and then inserted into the corresponding sites of the pET-24a(+) vector (Merck Millipore, Billerica, MA). The resultant plasmid was designated as pET-\textit{dhbF A1}.

\textit{Escherichia coli} BL21-CodonPlus(DE3)-RIL (Stratagene, La Jolla, CA) was transformed with pET-\textit{dhbF A1}. The cells transformed with pET-\textit{dhbF A1} were grown in Luria-Bertani (LB) media containing kanamycin (50 \( \mu \)g/ml) and chloramphenicol (34 \( \mu \)g/ml) at 37\( ^\circ \)C. When the culture reached an optical density at 600 nm of approximately 0.6, the incubation temperature was reduced to 25\( ^\circ \)C, and protein expression was induced with 0.005 mM isopropyl-\( \beta \)-D-thiogalactoside (IPTG). After 24 h culture, the cells were harvested by centrifugation, washed twice, and then lysed with B-PER Bacterial Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA) containing 0.1 mg/ml lysozyme and 6 units/ml DNase I. After the cell lysis, the cell debris was removed by centrifugation (27,000 \( \times \)g, 30 min). The resulting supernatant was applied to a Ni-chelating column of HisTrap HP (5 ml) (GE Healthcare, Piscataway, NJ) and washed with 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl. DhbFA1 was eluted with a linear gradient (0.01–0.4 M) of imidazole. The fractions containing only the 60 kDa protein, the size of which corresponds to the amino acid sequence deduced from the nucleotide sequence of \textit{dhbF A1}, were collected and dialyzed against 20 mM Tris-HCl buffer (pH 7.5). The homogeneity of the purified proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This purified fraction was used in all experiments in this study.

**(ii) \textit{dhbF A2}**

The following pair of oligonucleotide primers was used for \textit{dhbF A2} amplification: 5’-AAAGGAATTC-GATTCTTATC-3’ (KpnI recognition site is underlined) and 5’-GATCTGCAGGATCC-3’ (XhoI recognition site is underlined, and His-Tag sequence is double underlined). The \textit{dhbF A2} PCR product was digested with KpnI and XhoI, and then inserted into the corresponding sites of the pCold IV vector (Takara Bio Inc., Shiga, Japan). The resultant plasmid was designated as pCold-\textit{dhbF A2}.

\textit{E. coli} BL21-CodonPlus(DE3)-RIL was transformed with pCold-\textit{dhbF A2}, and the transformed cells were grown in LB media containing ampicillin (200 \( \mu \)g/ml) and chloramphenicol (34 \( \mu \)g/ml) at 37\( ^\circ \)C. When the culture reached an optical density at 600 nm of approximately 0.6, the incubation temperature was reduced to 15\( ^\circ \)C, and protein expression was induced with 0.5 mM IPTG. After 24 h culture, the cells were harvested by centrifugation and then lysed with B-PER Bacterial Protein Extraction Reagent. The purification method for DhbFA2 (62 kDa) was the same as that for DhbFA1 (60 kDa).

**Fig. 3.** Multiple sequence alignment of internal adenylation domains and enzymes belonging to the superfamily of adenylate-forming enzymes. Sequences were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and the identical or similar amino acids are shaded by BoxShade (https://embnet.vital-it.ch/software/BOX_form.html). Amino acids involved in substrate specificity (Challis et al., 2000; Röttig et al., 2011; Stachelhaus et al., 1999) for glycine or L-threonine are boxed. DhbE, the stand-alone adenylation domain in bacillibactin synthetases of \textit{B. subtilis}; EntE, the stand-alone adenylation domain in enterobactin synthetases of \textit{E. coli}; DltA, the \( \alpha \)-alanine: \( \alpha \)-amyl carrier protein ligase of \textit{B. subtilis}; DhbFA1 and DhbFA2, the internal adenylation domains expressed and analyzed in this study.
N-Acylation by adenylation domains and identification of the reaction products (dipeptides). The reaction mixture, comprising 100 mM glycine or l-threonine, 100 mM l-cysteine, 20 mM ATP, 8 mM MgCl₂, 100 mM (NH₄)₂SO₄, and 0.5 mg/ml of the purified DhbFA1 or DhbFA2 in 100 mM Tris-HCl buffer (pH 8.0 for DhbFA1 or pH 8.5 for DhbFA2), was incubated at 30°C, and the reaction was stopped by adding formic acid (final 0.7%, pH 3.0) to the reaction mixture.

The reaction mixture with DhbFA1 was then analyzed by HPLC on a CROWNPAK CR(+) column (4.0 × 150 mm; Daicel Chemical Industries, Ltd., Osaka, Japan). Perchloric acid (pH 1.5) was used as the mobile-phase solvent, and chromatographic separation was performed at 25°C in the flow rate of 1.0 ml/min. A new product peak (retention time, 3.7–3.9 min) other than that of AMP was detected by monitoring the column effluent at 190 nm. For LC-MS/MS analysis, the new product peak was collected, and then analyzed with a Shimadzu LCMS-8030 equipped with a Shimadzu Nexera HPLC system and a Develosil RPAQUEOUS column (2.0 × 150 mm; Nomura Chemical Co., Ltd., Aichi, Japan). The mobile-phase solvent was 5% (v/v) acetonitrile containing 0.1% (v/v) formic acid in water, and chromatographic separation was performed at 25°C in the flow rate of 0.2 ml/min. The MS/MS analysis data were acquired under the following conditions: curved desolvation and heat block temperatures of 300 and 200°C, respectively. Nitrogen was used as the nebulizer gas and drying gas at flow rates of 2 and 15 liters/min, respectively. The collision energy was varied from –35 to 35 V, and the ion source polarity was set in the positive or negative mode.

In the case of the reaction with DhbFA2, the reaction mixture was analyzed by HPLC on a Develosil RPAQUEOUS column (4.6 × 250 mm). The mobile-phase solvent was 5% (v/v) acetonitrile containing 0.1% (v/v) formic acid in water, and chromatographic separation was performed at 25°C in the flow rate of 1.0 ml/min. A new product peak (retention time, 5.3 min) other than that of AMP was detected by monitoring the column effluent at 193 nm. The MS/MS analysis data were collected under the same conditions as for the identification of the product of the DhbFA1 reaction.

Enzyme assays. Amide bond-synthetic activity was assayed by measuring acid-dependent AMP formation. Although the ATP-[³²P]PPi exchange assay (Lee and Lipman, 1975) or the colorimetric assay (Katano et al., 2012) has been used for measuring the activities of adenylyating enzymes, these assays involve indirect quantification of acyl-AMP or pyrophosphate (PPi). On the contrary, the modified method used in our study to directly measure the amount of AMP by HPLC (Kimura et al., 2004) allows easier and more accurate quantification than the methods reported previously. The reaction, stopped by adding an equal volume of methanol to the reaction mixture, was analyzed by HPLC. AMP and ATP in each reaction series were separated on a TitanSphere Tio HPLC column (4.6 × 150 mm; GL Science Inc., Tokyo, Japan). The mobile-phase solvent comprised 60 mM potassium phosphate buffer (pH 7.0) containing 60% (v/v) acetonitrile, and chromatographic separation was performed at 40°C in the flow rate of 1.0 ml/min, with monitoring at 260 nm. One unit was defined as the amount of enzyme that catalyzed the formation of 1 μmol AMP/min under these assay conditions.

To determine the kinetic parameters of the DhbFA1 reaction, the reaction mixture comprising 5–100 mM l-glycine, 5–150 mM l-cysteine, 20 mM ATP, 8 mM MgCl₂, 100 mM (NH₄)₂SO₄, and 0.5 mg/ml of the purified DhbFA1 in 100 mM Tris-HCl buffer (pH 8.5), was incubated at 50°C for 40 min. To determine those of the DhbFA2 reaction, the reaction mixture comprising 10–250 mM l-threonine, 0.0005–0.8 mM l-cysteine, 20 mM ATP, 8 mM MgCl₂, 100 mM (NH₄)₂SO₄, and 0.5 mg/ml of the purified DhbFA2 in 100 mM Tris-HCl buffer (pH 8.5), was incubated at 30°C for 120 min. $K_m$ and $V_{max}$ values were obtained using SigmaPlot 12.5 (Hulinks Inc., Tokyo, Japan) software. $k_{cat}$ values were calculated using 59.8 and 62.3 kDa for DhbFA1 and DhbFA2, respectively.

Substrate specificity. The amino acids [100 mM for other amino acids than the following: l-leucine (50 mM), l-asparagine (50 mM), l-phenylalanine (50 mM), l-glutamic acid (25 mM), l-aspartic acid (12.5 mM), l-tyrosine (6.25 mM), l-tryptophan (6.25 mM), D-aspartic acid (6.25 mM), D-tyrosine (6.25 mM), and l-tryptophan (6.25 mM)] and dipeptides (100 mM) listed in Subsection “Materials” were examined instead of glycine or l-threonine as to substrate specificity for the synthesis of aminocacyl-l-cysteine of DhbFA1 or DhbFA2, respectively. In order to investigate the substrate specificity for cysteine side oligopeptides, we used 5 mM oligopeptides listed in Subsection “Materials”. The increase in the amount of AMP during the enzymatic reaction was determined by HPLC using the assay described in Subsection “Enzyme assays”.

Analytical methods. Protein concentrations were measured with a Nakalai Tesque protein assay kit with bovine serum albumin as the standard. SDS-PAGE was performed in an 11.5% polyacrylamide slab gel. The gel was stained with Coomassie brilliant blue R-250. The relative molecular mass of the enzyme was calculated from the mobilities of the marker proteins: α-2-macroglobulin (180 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (79 kDa), aldolase (42 kDa), carbonic anhydrase II (30 kDa), trypsin inhibitor (20 kDa), and lysozyme (14 kDa).

Results

Preparation of DhbFA1, one internal adenylation domain in DhbF and identification of the reaction product.

Because there have been no reports that the internal adenylation domains of DhbF alone are expressed and characterized, the gene regions of internal adenylation domains of DhbF have remained unclear. Therefore, we tried to express each of the dhbFAs based on multiple sequence alignment (Fig. 3). An expression plasmid, pET-dhbFA1, was constructed and introduced into E. coli BL21-CodonPlus(DE3)-RIL. After the E. coli transformant had been cultured for 24 h at 24°C, a cell-free extract was prepared. C-Terminally histidine$_6$-tagged DhbFA1 (543 amino acid residues) was simply purified by Ni-chelating col-
aminoacyl- L-cysteine (dipeptide) synthetic reaction or not, nally histidine 6-tagged DhbFA2 (562 amino acid residues)∞

The transformant was cultured for 24 h at 15 C. C-Terminal histidine 6-tagged DhbFA2 (62 kDa) was confirmed by SDS-PAGE (Fig. 4, lane 2).

We examined whether DhbFAs, adenylation domains in the DhbF multidomain, could also catalyze the N-aminoacyl-L-cysteine (dipeptide) synthetic reaction or not, when L-cysteine was used as a substrate. Since DhbFA1 originally uses glycine and 4’-phosphopantetheine in the PCP domain of DhbF as substrates, a reaction mixture comprising DhbFA1, glycine, L-cysteine, and ATP was analyzed. After stopping the reaction, the reaction mixture was applied to a CROWNPAK CR(+)- column and identified by the enzyme in the reaction mixture, the peaks derived from the new product and AMP were not detected. The product peak was collected and then subjected to LC-MS/MS analysis. The determined molecular mass of the product with a retention time of 5.3 min (Fig. 5B) was determined to be 222 Da, which is consistent with the molecular mass of N-L-threonyl-L-cysteine (Thr-Cys) or S-L-threonyl-L-cysteine. Together with this finding, a fragment ion at m/z 143 (Fig. 6C) derived from the precursor ion at m/z 221 in the negative ion mode indicated that the product is Thr-Cys, but not S-L-threonyl-L-cysteine.

Characteristics of dipeptide synthetic activity of DhbFAs
Because the above dipeptide syntheses are accompanied by the reaction ATP → AMP + PPi, i.e. a decrease in the amount of ATP and an increase in that of AMP, we determined the amount of AMP by HPLC to evaluate the enzyme activities. The resultant reaction mixture was analyzed by HPLC on a TitaSphere TiO HPLC column with authentic AMP as a standard.

The Kₘ and Vₐₘₐₓ values of the dipeptide syntheses, calculated from Michaelis-Menten kinetics (Fig. 7), are shown in Table 1. Although the Vₐₘₐₓ values of DhbFA1 and DhbFA2 were not very different from each other (DhbFA1, 0.00735 units/mg; DhbFA2, 0.00373 units/mg), there was a significant difference between the Kₘ values for L-cysteine of DhbFA1 (21.5 mM) and DhbFA2 (0.0163 mM).

The effects of temperature and pH on the dipeptide synthetic reactions were also examined. The optimal reaction temperature of DhbFA1 for Gly-Cys synthesis appeared to be 50°C (Fig. 8A, left) when the reaction was carried out for 30 min. In contrast, that of DhbFA2 for Thr-Cys synthesis appeared to be 20°C, as shown in Fig. 8A (right). The maximum activity of DhbFA1 was seen at pH 8.5 and that of DhbFA2 in the pH range of 8.5–9.0, as shown in Fig. 8B.

Reactivities for other amino acids
Instead of glycine or L-threonine, another amino acid or

was purified through the purification steps described in Section “Materials and Methods”. The purity of DhbFA2 (62 kDa) was confirmed by SDS-PAGE (Fig. 4, lane 2).

For the determination of the product of the DhbFA2 reaction, a reaction mixture comprising DhbFA2, L-threonine, L-cysteine, and ATP was applied to a Develosil RPAQUEOUS column on an LC-MS/MS system. The molecular mass of the product peak with a retention time of 20.5 min (Fig. 5B) was determined to be 222 Da, which is consistent with the molecular mass of N-L-threonyl-L-cysteine (Thr-Cys) or S-L-threonyl-L-cysteine. Together with this finding, a fragment ion at m/z 143 (Fig. 6C) derived from the precursor ion at m/z 221 in the negative ion mode indicated that the product is Thr-Cys, but not S-L-threonyl-L-cysteine.

Table 1. Kinetics comparison between DhbFA1 and DhbFA2.

| Kinetic parameters | DhbFA1 | DhbFA2 |
|--------------------|--------|--------|
| Vₐₘₐₓ (U/mg)       | 0.00735 ± 0.000309 | 0.00373 ± 0.000284 |
| Kₘ for L-cysteine (mM) | 21.5 ± 5.28 | 0.0163 ± 0.00460 |
| kₐ/Kₘ for L-cysteine (sec⁻¹·mM⁻¹) | 0.341 | 238 |
| Kₘ for amino acids (mM) | 17.2 ± 2.35 (for glycine) | 105 ± 18 (for L-threonine) |
| kₐ/Kₘ for amino acids (sec⁻¹·mM⁻¹) | 0.426 (for glycine) | 0.0369 (for L-threonine) |

The reactions were carried out in triplicate with measurement of acid-dependent AMP formation as described in Section “Materials and Methods”.

Preparation of DhbFA2, the other adenylation domain in DhbF, and identification of the reaction product
An expression plasmid, pCold-dhbFA2, was constructed and introduced into E. coli BL21-CodonPlus(DE3)-RIL. The transformant was cultured for 24 h at 15°C. C-Terminally histidine 6-tagged DhbFA2 (562 amino acid residues) was purified through the purification steps described in Section “Materials and Methods”. The purity of DhbFA2 (62 kDa) was confirmed by SDS-PAGE (Fig. 4, lane 2).

Fig. 4. SDS-PAGE of the purified DhbFA1 and DhbFA2. Protein bands were detected by staining with Coomassie brilliant blue. Lane M, marker proteins; lane 1, the purified DhbFA1 (0.5 µg); lane 2, the purified DhbFA2 (0.5 µg).

| seawater chromatography as described in Section “Materials and Methods”. The purity of DhbFA1 was confirmed by migration of each of the proteins as a single band corresponding to a molecular mass (60 kDa) on SDS-PAGE (Fig. 4, lane 1).

Fig. 4. SDS-PAGE of the purified DhbFA1 and DhbFA2.

Protein bands were detected by staining with Coomassie brilliant blue. Lane M, marker proteins; lane 1, the purified DhbFA1 (0.5 µg); lane 2, the purified DhbFA2 (0.5 µg).
Peptide synthesis by adenylation domains

Dipeptide was added to the reaction mixture, and the ability of DhbFA1 or DhbFA2 to produce a peptide was measured as the amount of AMP produced. In the absence of DhbFA1 or DhbFA2 in the reaction mixture, an increase in the amount of AMP was not detected. Among the tested L-amino acids or dipeptides listed in Section “Materials and Methods”, a significant increase in AMP was observed with L-serine (relative activity, 21%) or L-aspartic acid (relative activity, 5%), when DhbFA1 was used (Table 2). The formation of N-L-seryl-L-cysteine (Ser-Cys) was also confirmed by LC-MS/MS (Fig. 6B). The tested dipeptides were inert as substrates. In the case of DhbFA2, all L-amino acids except L-serine or L-aspartic acid were not substrates for DhbFA2.

Fig. 5. HPLC chromatograms of the reaction mixtures.
A. HPLC chromatograms of the reaction mixture including glycine, L-cysteine, and DhbFA1 (CROWNPAK CR(+) column). B. HPLC chromatograms of the reaction mixture including L-threonine, L-cysteine, and DhbFA2 (Develosil RPAQUEOUS column). 0 h or 8 h indicates the reaction time. Authentic indicates the purchased authentic Gly-Cys.

Fig. 6. MS/MS spectra of dipeptides formed by DhbFA1 or DhbFA2 in the negative ion mode.
A and B. MS/MS spectra of Gly-Cys (A) or Ser-Cys (B) formed by DhbFA1. C. MS/MS spectra of Thr-Cys formed by DhbFA2. The structural formulae show Gly-Cys (A), Ser-Cys (B), or Thr-Cys (C). The theoretical m/z values of the fragments are provided with the structure. The fragment ion at m/z 99 (A) suggests that the product is not S-glycyl-L-cysteine but Gly-Cys; m/z 129 (B) suggests that the product is not S-L-seryl-L-cysteine but Ser-Cys; and m/z 143 (C) suggests that the product is not S-L-threonyl-L-cysteine but Thr-Cys. Authentic indicates the purchased authentic Gly-Cys.
Fig. 7. Michaelis-Menten plots of the dipeptide synthetic reactions. A. Michaelis-Menten plots of the Gly-Cys synthetic reaction by DhbFA1. B. Michaelis-Menten plots of the Thr-Cys synthetic reaction by DhbFA2.

Fig. 8. Effects of temperature and pH on the dipeptide synthesis by DhbFA1 or DhbFA2. Relative activity is expressed as a percentage of the maximum activity attained under the experimental conditions used. A. The reactions were carried out for 30 min at various temperatures. B. The reactions were carried out for 60 min at 50°C for DhbFA1, or 20°C for DhbFA2. In the pH profile experiment on DhbFA1, a three-component buffer system (Ellis and Morrison, 1982) was used. Buffer A consisted of 50 mM acetic acid, 50 mM MES, and 100 mM Tris (H17033), and buffer B consisted of 100 mM ACES, 52 mM Tris, and 52 mM ethanolamine (H17034). In the case of DhbFA2, the reactions were carried out in the following buffers (0.1 M): PIPES (●), Tris-HCl (○), and borate-NaOH (■).

Reactivities for oligopeptides (cysteine side)

We also observed an increase in the amount of AMP in the reaction mixture containing D-cysteine, L-cysteinylglycine (Cys-Gly), Cys-Gly-Gly-Arg-Glu, or Cys-Gly-Gly-Arg-Glu-Ser-Gly-Ser instead of L-cysteine as a substrate (Table 3). In the absence of DhbFA1 or DhbFA2 in the reaction mixture, an increase in the amount of AMP was not detected. When the concentration of each of the substrates was 5 mM, the activities of DhbFA1 and DhbFA2 for D-cysteine, Cys-Gly, and these oligopeptides were almost the same (more than 72%) as that for L-cysteine.

Discussion

The adenylation domains of NRPSs are included in the superfamily of adenylate-forming enzymes (Conti et al., 1996; Ehmann et al., 2000; Gulick, 2009). We previously found a novel N-acylation reaction for an acyl-CoA synthetase, AcsA (Hashimoto et al., 2005), which is also a member of the superfamily. Although acyl-CoA synthetases catalyze the ligation of an acid with CoA, N-acyl-L-cysteine is surprisingly synthesized, when L-cysteine is used as the substrate instead of CoA (Abe et al., 2008). This new N-acylation enzyme activity was also acids or dipeptides, other than L-threonine, were inert. Furthermore, DhbFA1 and DhbFA2 exhibited no detectable activity toward D-amino acids.
confirmed for DltA (Abe et al., 2016) and DhbE (Abe et al., 2017) of *Bacillus subtilis*, two of which belong to the same superfamily of adenylylate-forming enzymes. Recently, we clarified the mechanism of this unexpected reaction using DltA, which is the d-alanine:d-alanyl carrier protein ligase (Kiriukhin and Neuhaus, 2001; Yonus et al., 2008). The proposed mechanism for the peptide/amide synthesis involving DltA is as follows: (i) the formation of S-acyl-L-cysteine as an intermediate through its enzymatic catalysis, and (ii) a subsequent spontaneous rapid $S \rightarrow N$ acyl shift in the intermediate to allow peptide/amide bond formation (Abe et al., 2016). Like DltA, DhbFA1 and DhbFA2 belong to the superfamily of adenylylate-forming enzymes. Therefore, the reaction mechanism of dipeptide synthesis by these enzymes would be the same as that by DltA.

Peptide syntheses catalyzed by the above $N$-acylation reaction of adenylation domains have never been previously reported. Here, we first succeeded in the expression and purification of each of the DhbF “internal” adenylation domains (DhbFA1 and DhbFA2) separately, and further demonstrated that the internal adenylation domains synthesized dipeptides through the $N$-acylation reaction; DhbFA1 or DhbFA2 synthesized Gly- DL-Cys or L-Thr-DL-Cys, respectively (Figs. 2B and 2D “Dipeptide synthetic reaction” in dotted boxes).

There was a significant difference between the $K_m$ values for L-cysteine of DhbFA1 and DhbFA2 (Table 1). While the $K_m$ value of DhbFA1 for L-cysteine (21.5 mM) was higher than that of DhbFA2 (0.0163 mM), it was lower than that of DhbE (150 mM) (Abe et al., 2017). The significant difference in the $K_m$ values for L-cysteine of these adenylation domains is inferred to reflect the $K_m$ values for the 4′-phosphopantetheine arm of the corresponding PCP domains. Although DhbFA1 and DhbFA2 originally are part of a multiple domain, DhbFA1 or DhbFA2 separately was artificially expressed in this study. DhbFA1 or DhbFA2 originally is part of the multiple domain enzyme DhbF and they adjoin PCP domains tethered 4′-phosphopantetheine, which is a substrate of DhbFA1 or DhbFA2. Therefore, DhbFA1 or DhbFA2 would not use cysteine as the substrate in vitro. Even though cysteine is not an essential substrate for the adenylation domains, the $K_m$ value for L-cysteine of DhbFA2 was low (0.0163 mM). The low $K_m$ value has advantages in the production of peptides.

Although an increase in the amount of AMP was detected when, instead of glycine, L-serine or L-aspartic acid was added to the reaction mixture containing DhbFA1, the catalytic activity toward these amino acids was low (Table 2). As for DhbFA2, it showed catalytic activity toward L-threonine only. The “stand-alone” adenylation domain DhbE uses some aromatic and straight-chain fatty acids as substrates (Abe et al., 2017). The d-alanine:d-alanyl carrier protein ligase DltA uses some d-amino acids and a few L-amino acids (Abe et al., 2016). In comparison with these enzymes, the “internal” adenylation domains in the multidomain enzyme may have narrow substrate specificities.

DhbFA1s also synthesized oligopeptides using Cys-Gly, Cys-Gly-Gly-Arg-Glu, or Cys-Gly-Gly-Arg-Glu-Ser-Gly-Ser-Gly-Ser instead of L-cysteine. Their tripeptide, hexapeptide, and undecapeptide synthetic activities were also similar (more than 72%) to the dipeptide (Gly-Cys or Thr-Cys) synthetic activity (Table 3), suggesting that synthesis of oligopeptides by DhbFA1s is independent of the length of the peptide containing the N-terminal cysteine residue. In other words, DhbFA1s can synthesize oligopeptides longer than an undecapeptide in almost the same yield as for dipeptide synthesis.

In bacillibactin biosynthesis, a peptide compound (DHB-Gly-Thr) is formed (Fig. 1). However, at least three domains [i.e., adenylation domain, PCP domain, and condensation domain] are required to form a peptide/amide bond. For example, DhbFA1 catalyzes the binding of glycine to the PCP domain in DhbF. The reaction for peptide bond formation between DHB bound to the PCP domain and glycine bound to the other PCP domain proceeds under the catalytic control of the condensation domain in DhbF (Trauger et al., 2000). Furthermore, for the release of the final product from DhbF, a thioesterase domain with hydrolytic cleavage activity is needed. In other words, an NRPS system requiring at least four domains to produce a peptide/amide compound is very complicated. On the other hand, our new peptide/amide compound synthesis with the adenylation domain only is very simple.

### Table 2. Substrate specificity for amino acids.

| Amino acids | Relative activity (%) DhbFA1 | Amino acids | Relative activity (%) DhbFA2 |
|-------------|-----------------------------|-------------|-----------------------------|
| Glycine     | 100                         | L-Threonine | 100                         |
| L-Serine    | 21                          |             |                             |
| L-Aspartic acid | 5                           |             |                             |

The reactions were carried out in triplicate with measurement of acid-dependent AMP formation as described in Section “Materials and Methods”.

### Table 3. Substrate specificity for oligopeptides (cysteine side).

| Cysteine or cysteine-containing oligopeptides | Relative activity (%) DhbFA1 | DhbFA2 |
|---------------------------------------------|------------------------------|--------|
| L-Cysteine                                  | 100                          | 100    |
| ß-Cysteine                                  | 96                           | 96     |
| Cys-Gly                                     | 107                          | 78     |
| Cys-Gly-Gly-Arg-Glu                         | 98                           | 72     |
| Cys-Gly-Gly-Arg-Glu-Ser-Gly-Ser-Gly-Ser      | 108                          | 78     |

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### Materials and Methods

Peptide synthesis by adenylation domains

The reactions were carried out in triplicate with measurement of acid-dependent AMP formation as described in Section “Materials and Methods”.

Confined for DltA (Abe et al., 2016) and DhbE (Abe et al., 2017) of *Bacillus subtilis*, two of which belong to the same superfamily of adenylylate-forming enzymes. Recently, we clarified the mechanism of this unexpected reaction using DltA, which is the d-alanine:d-alanyl carrier protein ligase (Kiriukhin and Neuhaus, 2001; Yonus et al., 2008). The proposed mechanism for the peptide/amide synthesis involving DltA is as follows: (i) the formation of S-acyl-L-cysteine as an intermediate through its enzymatic catalysis, and (ii) a subsequent spontaneous rapid $S \rightarrow N$ acyl shift in the intermediate to allow peptide/amide bond formation (Abe et al., 2016). Like DltA, DhbFA1 and DhbFA2 belong to the superfamily of adenylylate-forming enzymes. Therefore, the reaction mechanism of dipeptide synthesis by these enzymes would be the same as that by DltA.

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The usefulness of functional oligopeptides has been stud-
ied in diverse areas, such as pharmaceutical, health food, and cosmetic fields (Grotteli et al., 2016; Mills et al., 2011; Santos et al., 2012). Therefore, new methods for the synthesis of oligopeptides using enzymes have received much attention (Arai et al., 2013; Kino and Kino, 2015; Tanaka et al., 2017). In this study, we found that DhbFA1 and DhbFA2, which are the adenylation domains in the multidomain enzyme of NRPS, synthesize dipeptides. Because many adenylation domains that could activate the respective substrates (Finkling and Marahiel, 2004; Labby et al., 2015; Roongsawang et al., 2011) are present in the natural world, we should be able to synthesize various peptides or amides by using adenylation domains or enzymes belonging to the superfamily of adenylate-forming enzymes.

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