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A Putative Peptide Synthetase from *Bacillus subtilis* 713 Recognizing L-Lysine, L-Tryptophan, and L-Glutamic Acid

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Abstract Peptide synthetases produced from various microorganisms are multifunctional enzyme complexes and their substrates are recognized and activated by adenylation domains. To identify the substrate specificity of the peptide synthetase isolated from *Bacillus subtilis* 713, known to produce an antifungal peptide, two adenylation domains containing the minimal functional portion were expressed and purified. ATP-ppi exchange experiments and kinetic studies revealed that the two adenylation enzymes had a substrate specificity to L-lysine and L-tryptophan, respectively. In addition, based on a signature sequence comparison, the substrate of the third domain was predicted to be L-glutamic acid. These results suggest that this peptide synthetase is novel because there has been no previous report on a peptide synthetase that uses L-lysine, L-tryptophan, and L-glutamic acid as substrates in that order.

Key words: Adenylation domain, ATP-ppi exchange, *Bacillus subtilis*, peptide antibiotics, substrate specificity

Many biologically active peptides produced by bacteria and fungi are synthesized by nonribosomal peptide synthetases. These peptides have potent biological qualities, including antibacterial (vancomycin), antitumor (bleomycin), and immunomodulator (cyclosporins) properties, etc. [2, 13, 21]. Moreover, these families of antibiotics have 20 proteinogenic amino acids plus unusual amino acids with modified structures [5]. Despite of their diverse structures and origins, most of these peptides share a common mode of synthesis called a protein controlled thiotemplate mechanism, in which an individual enzyme catalyzes the peptide bond formation [6, 8, 13].

Genetic and biochemical analyses have revealed that various peptide synthetases have an iterative modular structure and sequences that are well conserved. A minimal module is composed of and characterized by a set of short conserved sequence motifs named adenylation, thiolation, and condensation domains. An adenylation domain is the most important part of a peptide synthetase as it bears the substrate recognition pocket and activates the specific substrate amino acid as an amino acyladenylate at the expense of ATP. This amino acyladenylate is subsequently tethered onto the cofactor 4-phosphopantetheine at the conserved serine residue of a thiolation domain. A condensation domain is responsible for the condensation of two activated peptidyl (or aminoacyl-) moieties [13, 19, 27].

The current authors previously isolated and reported on the gene involved in the synthesis of the peptide antibiotic from *Bacillus subtilis* 713 that is known to produce an antifungal cyclic peptide [10, 11, 17]. Three amino acid-activating domains were identified by sequence analysis [9, 18]. The peptide synthetase encoded by this gene has a typical modular structure as recognized in peptide synthetases belonging to a nonribosomal origin. The present report describes the identification of the substrate specificity and biochemical characteristics of the three amino-acid-activating domains of this peptide synthetase. The three successive adenylation domains exhibited a substrate specificity to L-lysine, L-tryptophan, and L-glutamic acid based on a signature sequence comparison. This suggested that this peptide synthetase is responsible for the synthesis of a novel peptide antibiotic, as there has been no previous report on a peptide antibiotic containing 4 valines and L-lysine, L-tryptophan, and L-glutamic acid in that order.

Materials and Methods

Microorganisms, Plasmids, DNA Manipulation

Escherichia coli DH5αF' and *E. coli* BL21 (DE3) were used as the hosts for the plasmid constructions and heterologous

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expression of each adenylation module, respectively. A Luria-Bertani (LB) medium was used for the routine cultivation of *E. coli* DH5αF'. 2X YT was used for the expression of heterologous genes in *E. coli* BL21 (DE3). pSK80 and pC72 contain the 8 kb and 7.2 kb peptide synthetase fragments of *B. subtilis* 713, respectively [9, 18]. pDAI and pDAII are pET28a-based vectors containing the two amplified adenylation domains of the peptide synthetase. The molecular cloning and PCR procedures were carried out using standard techniques [23].

Chemicals

Most chemicals, including the amino acids, were purchased from Sigma Co. (St. Louis, U.S.A.). The [^{32}P]-pyrophosphate was purchased from NEN Life Science (Boston, U.S.A.).

PCR Amplification

The primers, synthesized by Genotech (Taeduk, Korea), were as follows. Some nucleotides indicated by bold characters were modified to introduce a restriction enzyme site for easy cloning: DAI-forward - *Eco*RI, 5'-GCACAGCATTT-TACT**GAATTC**GTACGTGCG-3'; DAI - reverse - *Sal*I, 5'-CGGC**GTCGAC**GCCGAGCACTCCGGCCCAT-3'; DAII - forward - *Sac*I, 5'-ATTCGGGTGCAA**GAGCTC**GATGT-GTTAA-3'; DAII - reverse - *Xho*I, 5'-G**CTCGAG**TTTC-ACTTCCACCCACGTTCGC-3'.

The amplification of each adenylation domain was performed using Taq DNA polymerase (Takara, Shiga, Japan), a Perkin-Elmer thermal cycler (Foster, U.S.A., model 480), and pSK80 and pC72 as the templates. The PCR condition was 35 cycles of denaturation (94°C for 60 sec), annealing (30°C for 90 sec), and elongation (72°C for 120 sec). The amplified products were purified using a QIAEXII gel extraction kit (QIAGEN, Germany) and then ligated into a pGEM-T cloning vector (Promega, Madison, U.S.A.). The amplified 1.9 kb and 1.7 kb PCR products were ligated into a pET-28a (+) vector (Novagen, Madison, U.S.A.) at designated restriction enzyme sites for heterologous expression and further purification. The in-frame construction was confirmed by DNA sequencing.

Overexpression of His_6-Tagged DAI and DAII Derivatives

The plasmids, pDAI and pDAII, containing the amplified PCR products of the adenylation domains, were transformed into *E. coli* BL21 (DE3). The transformants were inoculated into 3 ml of a 2×YT broth supplemented with kanamycin (30 μg/ml) and then grown at 30°C with moderate shaking. The overnight culture (0.25 ml) was transferred into 250 ml of same culture broth in a 2-l Erlenmeyer flask. The cells were grown at 28°C with moderate shaking until A_{600} reached 0.4–0.5, then IPTG was added to a final concentration of 1.0 mM. After 3 h of further incubation, the cells were harvested by centrifugation at 6,000 ×g for 10 min. The extent of expression was measured by comparing with a known amount of a standard on a SDS-polyacrylamide gel [14].

Purification of Adenylation Enzyme

The Ni^{2+}-affinity chromatography (Novagen) was carried out at 4°C according to the manufacturer's instructions. The cell pellets were resuspended in a sonication buffer (50 mM sodium phosphate, pH 8.0 at 25°C, 300 mM sodium chloride) at 3 volumes/g of wet weight. Subsequently, the samples were lysed by sonication using a Branson sonicator operating at a middle output. Thereafter, the cell debris was pelleted by centrifugation at 35,000 ×g for 60 min, then the supernatant was directly applied to a Ni^{2+}-charged chelating column, previously equilibrated with a supplemented buffer. The protein was eluted by applying imidazole gradients ranging from 100–350 mM in the sonication buffer. The samples were pooled and dialyzed against an assay buffer (50 mM HEPES, pH 7.8 at 25°C, 100 mM NaCl, 1 mM EDTA, 1 mM $MgCl_2$, and 2 mM dithioerythrol). The purified proteins were analyzed by 10% SDS-polyacrylamide gel electrophoresis, and the protein concentration was measured using the procedure of Bradford [1].

ATP-ppi Exchange and Kinetic Studies

The substrate specificity of the peptide synthetase was determined using the well-known method of ATP-ppi exchange, as described by Lee [16] and Marahiel [22, 26, 27]. The assay mixture contained 100 nmol of the enzyme, 1.0 mM ATP, 2.0 mM of 20 proteinogenic amino acids and L-ornithine, 50 mM HEPES, pH 7.8 at 25°C, 100 mM NaCl, 1 mM EDTA, 1 mM $MgCl_2$, 2mM dithioerythrol, 0.1 mM tetrasodium pyrophosphate, and 0.15 μCi of [^{32}P]-pyrophosphate in a total volume of 100 μl. The reaction started with the addition of the enzyme and [^{32}P]-pyrophosphate into a reaction buffer, and was allowed to proceed for 15 min at 37°C. Kinetic measurements of the ATP-[^{32}P]-pyrophosphate exchange were performed at varying substrate concentrations of 0.02–1.2 mM ATP and 0.02–2.0 mM amino acids.

Specificity Conferring Code and Phylogenic Studies

The internal adenylation domains of *B. subtilis* 713 were compared with various selected nonribosomal peptide synthetase A domains from the Blast P and Swiss Prot database. About two hundred amino acids between homologous regions A3 to A6 were multiply aligned using the Clustal W program [30] in an EMBL server, and a phylogenic tree was constructed using the same program except perfoming in GenomeNet with bootstrap option.

RESULTS AND DISCUSSION

Heterologous Expression and Purification of Adenylation Domains

To isolate the minimal extension of an amino-acid-activating domain, the truncated fragments of the peptide synthetase

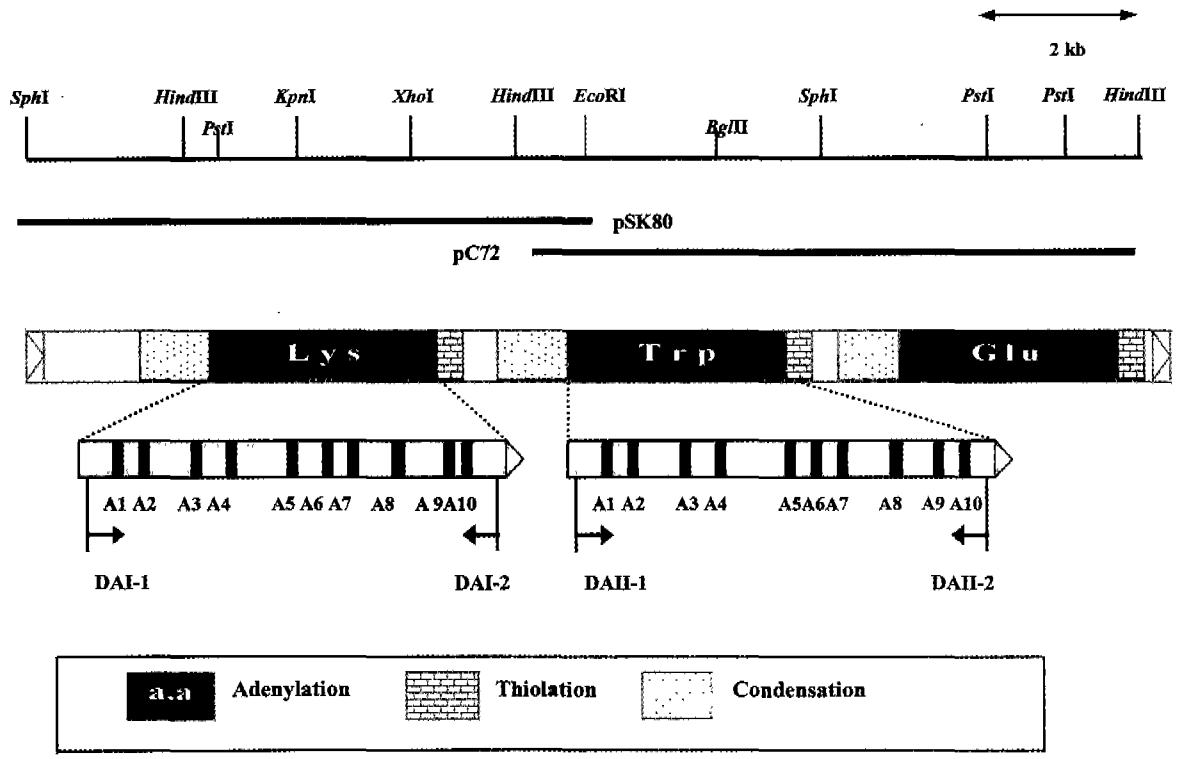

Fig. 1. Scheme for amplification of two adenylation domains.
Each primer was designed to amplify the regions A1 to A10 that are essential for the functions of substrate recognition and adenylate formation. pSK80 and pC72, containing the overlapped fragments of the peptide synthetase, were used as templates for the PCR.

gene from *B. subtilis* 713 were amplified. As shown in Fig. 1, each primer was designed to include the regions from A1 to A10 that are essential for substrate recognition and adenylation activities [22, 26, 27]. The first amplified fragment (DAI) was comprised of 1,926 base pairs and encoded a 73.5 kDa polypeptide, whereas the second amplified fragment (DAII) was comprised of 1,730 base pairs and encoded a 66 kDa polypeptide. Both proteins expressed were completely soluble. Moreover recombinant proteins, purified through Ni^{2+}-affinity column chromatography

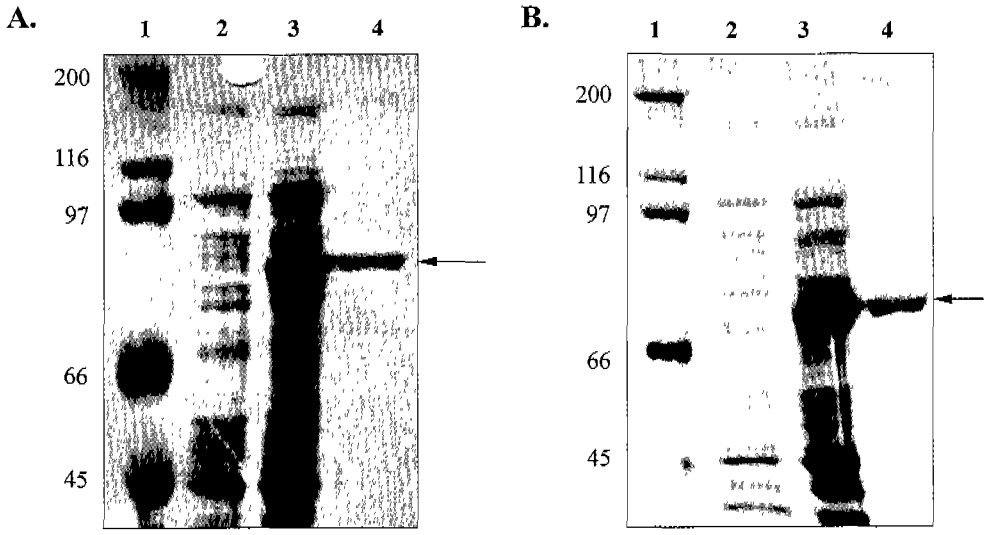

Fig. 2. SDS-polyacrylamide gels of *E. coli*-overexpressed two adenylation domains, DAI (A) and DAII (B).
Lane 1, broad range protein standards (purchased from Bio-Rad) from 200 kDa to 21.5 kDa; 2, whole-cell extracts prior to induction; 3, whole-cell extracts after 3 h induction with IPTG; 4, protein purified using Ni^{2+}-affinity chromatography. Two polypeptides of an adenylation domain are indicated by the arrow.

and analyzed by SDS-PAGE, were 74 kDa and 67 kDa polypeptides, respectively (Fig. 2).

Substrate Specificity of Adenylation Domains

The purified enzyme fractions containing an internal adenylation domain were analyzed by an amino-acid-dependent ATP-ppi exchange assay using all 20 proteinogenic amino acids and L-ornithine to determine the substrate specificity [16, 26, 27]. The first adenylation domain (DAI) activated lysine, whereas the second adenylation domain (DAII) activated tryptophan (Fig. 3). DAI also activated L-ornithine, yet with half the activity of that with L-lysine, and L-methionine slightly. Previously, based on other

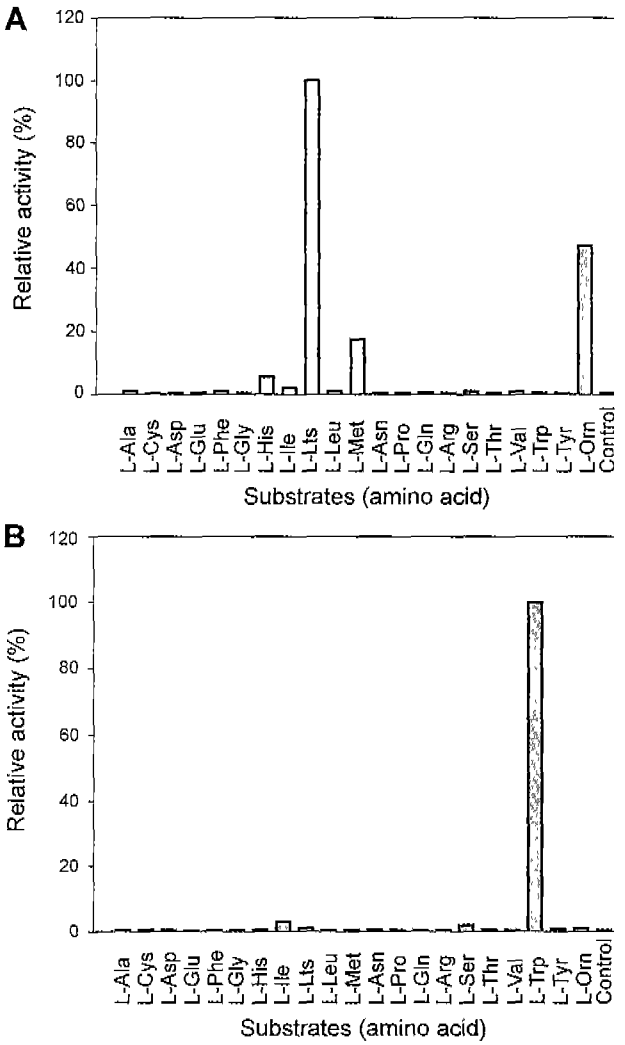

Fig. 3. Relative substrate specificity of purified proteins DAI (A) and DAII (B) by amino-acid-dependent ATP-ppi exchange experiments.

The activities are shown by bar graphs, and the highest activity is defined as 100%. Control experiments without any amino acid showed activities below 0.1%. DAI exhibited the highest recognition to lysine, half that to ornithine, and only some to methionine and histidine, whereas DAII showed a highly specific substrate recognition to tryptophan.

Table 1. Determination of K_m values for cognate amino acid substrates and ATP of internal adenylation domains of DAI and DAII.

Adenylation domain	Substrate	K_m (mM)	
		Amino acid	ATP
Lys	L-Lys	0.07±0.05	0.10±0.03
	L-Orn	0.12±0.05	0.27±0.16
Trp	L-Trp	0.07±0.05	0.8±0.2

sequence comparisons and phylogenic studies (Mootz, H. D., M. A. Marahiel, H.-J. Kwon, and J.-W. Suh, personal communication), the predicted substrate of the first domain was L- or D-ornithine. Because the sequence of three amino-acid-activating domains isolated from *B. subtilis* 713 lacks the epimerase domain, it was likely to select the L-form. To further define the substrate specificity of DAI, the quantitative affinities of the protein to their ATP substrate and the cognate amino acids (L-lysine and L-ornithine) were determined by measuring the K_m values (Table 1). The K_m value of DAI was lower with L-lysine than with L-ornithine, thereby suggesting that the substrate of the first domain (DAI) was L-lysine. This relaxed specificity has been found in other enzymes [22]. When compared with the ribosomal protein machinery, it is known that nonribosomal synthetases are less specific because they lack proofreading properties [7, 22]. Nonetheless, this fact can be beneficial for the structural diversity of nonribosomal peptide antibiotics in which more than 300 residues have been identified [5].

The substrate of the second adenylation domain (DAII) was apparently L-tryptophan, according to the ATP-ppi exchange and kinetic studies (Fig 3. and Table 1). Consistent with this result, a previous analysis of the antifungal peptide of *B. subtilis* 713 showed that it contains an aromatic amino acid. Although a complete structure of the antifungal peptide of *B. subtilis* 713 has not been elucidated, there would seem to be two active fractions, each predicted to have a similar number of constituent amino acids and sequence order [20]. Construction of the phylogenic tree and comparison based on multiple alignments of about 200 amino acids from regions A3 to A6 of the second adenylation domain with other peptide synthetases revealed that bootstrap values of the second adenylation enzyme significantly matched up to other aromatic amino-acid-activating enzymes (Fig. 4).

Prediction of Substrate of the Third Domain Based on Specificity Conferring Codes

The approach used to determine the substrate specificity of the third domain was sequence comparisons. The key amino acids involved in conferring the substrate specificity of peptide synthetases were recently proposed and assigned based on the X-ray crystallography of GrsA [3, 4, 26]. Comparisons based on multiple alignments of about 200

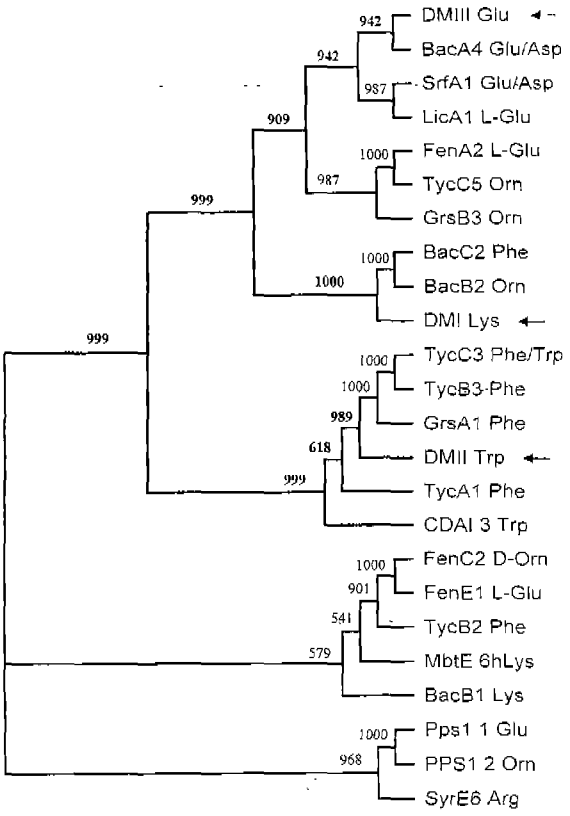

Fig. 4. Phylogenic tree based on alignments of amino acid sequences of 24 peptide synthetase modules from various microbial origins.
The numbers means the bootstrap value of each adenylation enzyme caculated using clustal W program [30]. To predict the substrate of the A-domain of the peptide synthetase from *B. subtilis* 713, approximately 200 amino-acid regions between the core sequence A3 and A6 (see Fig. 1.), which are thought to represent the substrate specificity-determining segment of a peptide synthetase module, were aligned using the clustal W program [30]. An amino acid specificity is represented by a three-letter amino acid code. The letters following the protein names represent the synthetase; the numbers represent the module within the subunit. The abbreviations of the protein names are as follows; Grs, gramicidine synthetase of *Bacillus brevis*; Tyc, tyrocidine synthetase of *Bacillus brevis*; Srf, surfactin synthetase of *Bacillus subtiis*; Fen, fengycin synthetase of *Bacillus subtiis*; Pps, plipastatin synthetase of *Bacillus subtilis*; Bac, bacitracin synthetase of *Bacillus licheniformis*, Lic, lichenysin synthetase of *Bacillus licheniformis*; Mbt, mycobactin synthetase of *Mycobacterium tuberculosis*; Syr, syringomycin synthetase of *Pseudomonas aeruginausa*.

amino acids from regions A3 to A6 of the third adenylation domain with other peptide synthetases revealed that 10 amino acids were completely conserved, as in other L-glutamic-acid-activating domains, thereby suggesting that its substrate was L-glutamic acid (Fig. 5). The known L-glutamic-acid-activating domains are conserved at the positions Trp239 and His278, however, in some cases, they are changed to Lys239 and Asp278, resulting in a broad specificity to both aspartic acid and glutamic acid [3]. This fact suggests that the substrate of the third domain was either glutamic acid or aspartic acid (Fig. 5). However, because the specific aspartate-activating modules were strictly

A-domain (substrate)	235 236 239 278 299 301 322 330 331 512
BacA (L-Glu)	D A K D I G V V D K
SrfA1 (L-Glu)	D A K D L G V V D K
Bs-DMIII(L-Glu)	**D A K D L G I V D K**

Fig. 5. Prediction of specificity of third adenylation domain.
The sequences between regions A3 to A6 of the third amino acid activating domain were aligned with other glutamic acid activating domains. Ten critical amino acid residues, called specificity conferring codes and known to form a substrate binding pocket, were aligned well with those of other non-ribosomal peptide synthetases [3]. The numbers indicate the positions of the amino acids that interacted directly with the substrate amino acid residues, as in GrsA [4]. The amino acid residues at positions 235, 236, 239, 278, 299, 301, 322, 330, 331, and 512 form the substrate binding pocket at the side chain of the substrate amino acid [3, 4, 26]. Each code is represented by a single letter amino acid code.

conserved at the positions Thr 239 and Lys 278, the substrate of the third domain was most likely glutamic acid [3]. Additionally, in the phylogenic analysis, the bootstrap value of the third domain nearly agreed with glutamic-acid-activiating domains of Surfactin, Lichenycin, and Bacitracin synthetase. The prediction of the specificity of peptide synthetases based on the sequence is very useful, however, the affinity to a predicted substrate is needed to be defined empirically.

Based on a biochemical and sequence analysis, it was concluded that the gene isolated from *B. subtilis* 713 is responsible for the biosynthesis of an antifungal peptide with the sequence L-lys-L-trp-L-glu. These results are consistent with previous structural data that suggest the presence of not only lysine and tryptophan but also glutamic acid. It was previously proposed that the three successive adenylation domains found in the peptide synthetase of *B. subtilis* 713 were L-lysine/ornithine-, L-tryptophan-, and glutamic-acid/aspartic-acid-activating domains, in that order.

A peptide containing the sequence of lysine-tryptophan-glutamic acid and 4 valines has not yet been reported, thereby suggesting that this peptide is most likely to be novel. In addition, the constituent amino acids of the peptide antibiotics from *B. subtilis* 713 are different from known cyclic decapeptide antibiotics, such as Cerexin [24], Fengycin [28], Gramicidin S [3, 8], Plipastatin [31], Polypeptin [25], AMRSA1 [15], YBS-1B [12], and Tyrocidine [22]. However, further studies on related genetic information and the peptide structure are still needed to prove the novelty of the peptide synthetase and the peptide from *B. subtilis* 713.

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