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Rapid and Efficient Isolation of Genes for Biosynthesis of Peptide Antibiotics from Gram-positive Bacterial Strains

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Abstract Peptide synthetases are large multifunctional enzyme complexes that catalyze the nonribosomal synthesis of a structurally diverse family of peptide antibiotics. These enzymes are composed of functionally independent domains with independent enzymatic activities. Their specific linkage order of domains forms the protein template that defines the sequence of the incorporated amino acids. Within each domain, several motifs of highly conserved sequences have been identified from the sequence alignment of the various peptide synthetases [30]. Taking advantage of the conserved nucleotide sequence of Core 1 and Core 2, we designed PCR primers to amplify the peptide synthetase genes from three different gram-positive bacterial strains. Nucleotide sequence analysis of the amplified PCR products from those three strains showed significant homology to various peptide synthetase genes, suggesting that the PCR products are parts of peptide synthetase genes. Therefore, this rapid and efficient PCR technique can be used for the isolation of peptide synthetase genes from various strains.

Key words: Peptide antibiotics, PCR, *Bacillus subtilis*, *Streptomyces*, nucleotide sequence

Since penicillin was discovered as a miraculous drug against bacteria, many antibiotics have been identified and developed using biological and chemical methods [31]. Among these, a family of peptide antibiotics has been studied and used widely in the pharmaceutical and agricultural industry because of its stability against various enzymes [28]. Peptide antibiotics belong to a group of antibiotics composed of a small number of peptides in linear or circular forms produced by several bacterial and fungal strains. Gram-positive bacteria, especially bacilli, are the extensively studied bacteria known to produce peptide antibiotics. These antibiotics contain nonprotein amino acid constituents which are highly modified by, for example, hydroxylation, acylation, *N*-methylation, and covalent linkage to other unusual functional groups [17, 31].

Peptide antibiotics are largely divided into two subgroups based on biosynthetic mechanism, namely, ribosomal biosynthesis and nonribosomal biosynthesis. Among the ribosomally-synthesized antibiotics produced by gram-positive bacteria, the lantibiotics [10, 27] have been studied in detail in terms of structure and the biochemical mechanism of their synthesis. These polypeptides are synthesized as precursors that undergo extensive processing and modification, resulting in a mature product of nineteen to thirty four amino acids. The lantibiotics have gained attention as potentially useful topical antibiotics and food additives [10, 27].

The other group of peptides are not synthesized by ribosomes, but rather by large multienzymes that use a thiotemplate mechanism [28]. Biochemistry of multienzyme-catalyzed synthesis of peptide antibiotics has been investigated in detail over the past thirty years [13, 14, 15, 28, 30]. These studies have revealed that distinct domains found in multienzymes represent the functional building units that are responsible for specific amino acid activation, modification, and peptide bond formation [15, 30].

Biosynthetic genes for peptide synthetases of bacterial and fungal origin required for nonribosomal synthesis of tyrocidine [20, 21, 32], gramicidine S [9, 16, 18], surfactin [7, 23, 24, 25], HC-toxin [30], enniatin A [30], cyclosporin A [30], and tripeptide precursor of β-lactam δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV, 3) were isolated and characterized. Sequencing and characterization of genes encoding multifunctional peptide synthetases have confirmed that the domains in the multifunctional enzymes are functional building units. The occurrence and specific order of each building unit dictate the

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number and sequence of the amino acids incorporated into the peptide product [30]. This fact has opened a new era in the development of noble antibiotics. For example, Stachelhaus *et al.* [29] developed noble antibiotics by genetic manipulation of domains of the peptide synthetase genes.

In addition, sequence alignment has revealed that the nucleotide sequence of peptide synthetase genes and the amino acid sequences of peptide synthetases are conserved enough to show six notable motifs (Core 1 to Core 6) of highly conserved sequences [30]. The function of the Core 1 motif is not known yet. Cores 2 to 5 are believed to be involved in ATP-binding and hydrolysis. The Core 6 motif seems to be involved in the catalysis of the formation of the thioester bond. In addition to these six cores which are found in the homologous region, the other motif (so-called spacer motif) is also found to be conserved in the relatively non-homologous regions. Among the conserved regions, Core 1 and Core 2 are the most highly conserved even in nucleotide sequences as well as in amino acid sequences [3]. These are located about 200 bp apart within one domain (Core 1 - 200 bp inner space - Core 2, Fig. 1). However, the distance between Core 2 and the next Core 1 in the downstream domain is somewhat different (hundreds to thousands) depending on the domains.

In this study, we designed PCR primers based on the highly conserved nucleotide sequence of Core 1 and Core 2 to isolate and characterize the peptide antibiotics synthetase genes from several strains. Since most peptide antibiotics are produced by gram-positive bacteria, we first focused on gram-positive bacteria. Chromosomal DNA of wild-type Marburg strain *Bacillus subtilis* PB2 and that of *Bacillus subtilis* 713 isolated from Korean soil known to produce antifungal compounds [11, 12, 22] were used as templates for polymerase chain reaction. The chromosomal DNA of *Streptomyces hygroscopicus* KCTC 9169 was also used as template for PCR to isolate a DNA fragment of peptide synthetase gene from a strain of actinomycetes. Nucleotide sequence analysis of amplified DNA fragments showed significant homology to various peptide synthetase genes, suggesting these are fragments of peptide synthetase genes.

MATERIALS AND METHODS

Bacterial Strains and Genetic Methods

Escherichia coli DH5αF' was used as the host for all plasmid constructions. *Bacillus subtilis* wild-type Marburg strain PB2 [4] was used for the isolation of the chromosome which contained the surfactin biosynthetase gene. Antifungal substance producing *Bacillus subtilis* 713 isolated from Korean soil was used in this study [11, 12, 22]. *Streptomyces hygroscopicus* KCTC 9169 was obtained from KCTC (Korean Collection for Type Cultures, Genetic Resources Center, Korea Institute of Science and Technology). Luria broth (LB) from Davis *et al.* [5] was used for routine growth of *E. coli* strains and *Bacillus subtilis*. Penassay broth (Antibiotic medium

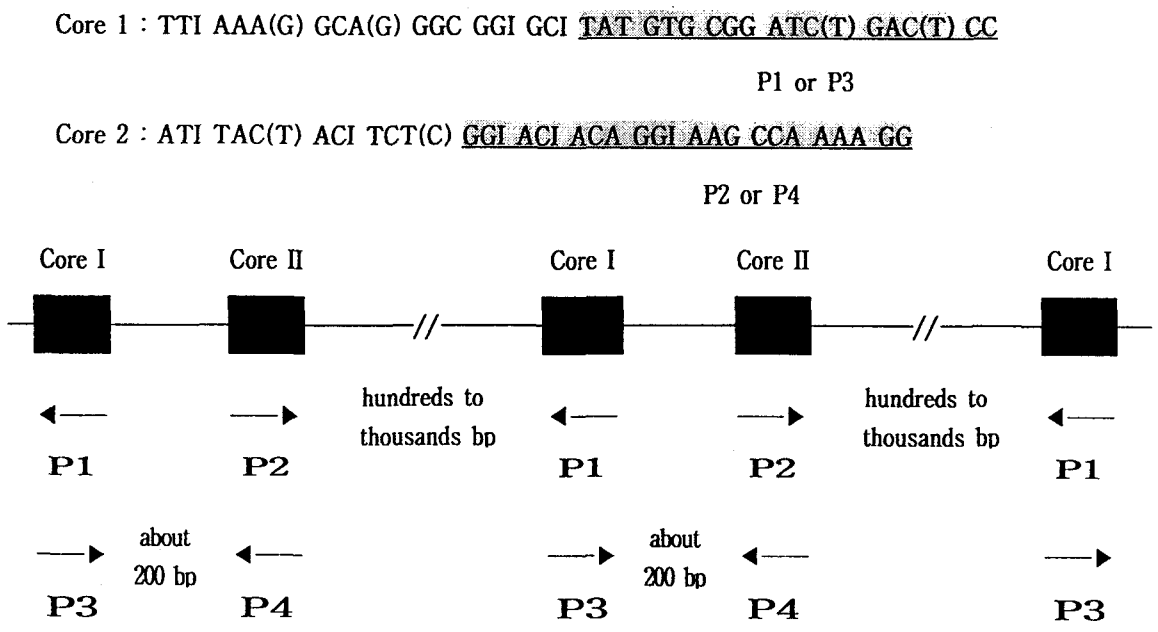

Fig. 1. Sequence of the primers used for polymerase chain reaction.

Primers 1 and 3 were designed based on the Core 1 sequence, primers 2 and 4 were designed based on the Core 2 sequence [27]. Primers 1 and 2 for PCR would give hundreds to thousands bp of PCR product, whereas primers 3 and 4 would give about 200 bp.

3, Difco Co., Detroit, U.S.A.) was used for growth of *Bacillus* strains for chromosome isolation.

DNA Methods and Nucleotide Sequencing

The suggested procedures of Davis *et al.* [5] and Sambrook *et al.* [26] were used for all standard recombinant DNA techniques. Most enzymes including restriction enzymes, ligase, Klenow, and *Taq* polymerase were purchased from Takara Co. Ltd. (Shiga, Japan). DNA sequencing was done by the dideoxynucleotide chain termination method for both double and single stranded plasmids using α-^{32}P-dATP (Amersham, Arlington Heights, U.S.A.) and the Sequenase version 2.0 enzyme as suggested by the manufacturer (US Biochemical, Cleveland, U.S.A.). Priming for DNA sequencing was done with custom oligonucleotide primers (Takara Co., Shiga, Japan and Bioneer Co., Daeduk, Korea) or with standard forward and reverse primers (US Biochemical, Cleveland, U.S.A.). Reaction conditions were as previously described [19]. Chromosomal DNA from the *Bacillus subtilis* and *Streptomyces hygroscopicus* were isolated by a modified version of the method of Ferrari *et al.* [6].

Polymerase Chain Reaction

In order to isolate the biosynthetic gene for peptide antibiotics from various strains, conserved regions — Core 1 and Core 2 — were used for designing the primers for polymerase chain reaction (Fig. 1). One primer set was primer 1: 5'-TAT GTG CGG ATC(T) GAC (T) CC-3' and primer 2: 5'- GGI ACI ACA GGI AAG CCA AAA GG-3'. The other primer set was primer 3: 5' GGG(A) TCG(A) ATC CGC ACA TA-3' and primer 4: 5'-CCT TTT GGC TTI CCT GTI GTI CC-3'. Polymerase chain reaction was performed using *Taq* polymerase (Takara Co., Shiga, Japan) and a Perkin-Elmer thermal cycler (model 480, Foster, U.S.A.) under the following conditions. Amplification was performed by 35 cycles of denaturation (94°C for 60 sec), annealing (30°C for 90 sec), and elongation (72°C for 120 sec). Amplified products were separated by agarose gel electrophoresis. The desired bands were eluted from the gel using the Qiaex II Agarose Gel Extract system (Qiagen, Chatsworth, U.S.A.) and then ligated into pGEM-T Easy vector (Promega, Madison, U.S.A.) which can be used for direct cloning of PCR product. The resulting plasmids were used for sequencing using pUC/M13 forward and reverse sequencing primers.

Computer Analysis

The statistical significance of predicted protein sequences was evaluated using the DNASIS (Hitachi Software Engineering, Japan) program, BLAST [1, 2], and FASTA (3.06 Sept., 1996) through the server system supported by NCBI. Highly related sequences usually have an optimized alignment score greater than 150 and z-value greater than 150. For multiple alignment between amino acids, the CLUSTAL V program [8] was used.

Results and Discussion

Optimization of Polymerase Chain Reaction

In order to isolate the biosynthetic genes for peptide antibiotics from the various bacterial strains, we decided to employ the polymerase chain reaction (PCR) using the conserved regions of the biosynthetic genes reported earlier [3]. Core 1 region whose function is not yet clear and Core 2 region which functions as an ATP binding site were used [27] for designing the PCR primers. One set of primers (primer 3 and primer 4, see Materials and Methods) was designed to amplify the regions between Core 1 and Core 2 of the same domain which should theoretically give about 200 bp-long PCR products. The other set of primers (primers 1 and 2, see Materials and Methods) should give several PCR products ranging from hundreds to thousands of bp from Core 2 of the upstream domain to the next Core 1 of the following domain (Fig. 1).

In order to determine whether the PCR primers could amplify the desired products, we used the chromosomal DNA from wild-type *Bacillus subtilis* strain PB2 [4] as a positive control because the nucleotide sequence of the surfactin synthetase gene from *Bacillus subtilis* had already been reported [7, 23, 24, 25]. Since we allowed the mismatch in the primer designing, we first tested the effects of PCR reaction temperature. Among the various annealing temperatures tested, we decided to use 30°C for the annealing temperature, because we could not obtain significant bands with annealing temperature over 37°C. Therefore, we decided to use 30 cycles of 94°C for 60 sec, 30°C for 90 sec, and 72°C for 120 sec for the PCR amplification of the biosynthetic gene. With 30°C as the annealing temperature, we obtained several bands including two major bands (bands 3 and 5 in lane 1, Fig. 2a) from wild-type *Bacillus subtilis* PB2.

Also, two sets of primers were compared in the PCR. Lane 1 in Fig. 2a shows that the primer set of primer 1 and primer 2 gives more than one band which we predicted because the distance between Core 2 of the upstream domain and the next Core 1 of the following domain varies depending on the domains. On the other hand, the other set of primers (primer 3 and 4) gives almost one band of about 250 bp which seems to contain heterogeneous products with the same size (lane 3, Fig. 2a), which is expected because most peptide antibiotics synthetase genes are composed of several domains and each domain has a relatively fixed distance of about 200 bp between Core 1 and Core 2 in the same domain. We

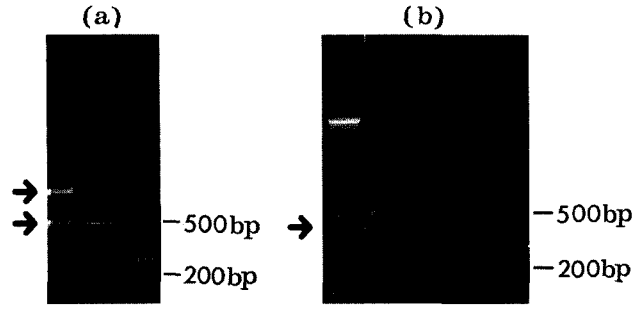

Fig. 2. Agarose gel electrophoresis of PCR products from various bacterial strains.

(a) lane 1: PCR products with primers 1 and 2 using wild-type *Bacillus subtilis* PB2 chromosomal DNA as template; Bands 3 and 5 are indicated (PB2-3 and PB2-5); lane 2, 100 bp ladder size marker, 200 bp and 500 bp are indicated; lane 3, PCR products with primers 3 and 4 using wild-type *Bacillus subtilis* PB2 chromosomal DNA as template. (b) lanes 1 and 2 are PCR products with primers 1 and 2 using *Bacillus subtilis* 713 chromosomal DNA (lane 1) and *Streptomyces hygroscopicus* 9169 chromosomal DNA (lane 2); lane 3, 100 bp ladder size marker, 200 bp and 500 bp are indicated; lanes 4 and 5, PCR products with primers 3 and 4 using *Bacillus subtilis* 713 chromosomal DNA (lane 4) and *Streptomyces hygroscopicus* 9169 chromosomal DNA (lane 5) as templates.

found that the two primer sets were equally useful in amplifying the peptide antibiotics synthetase genes from the nucleotide sequencing analysis (data not shown). However, we mostly used primer sets 1 and 2 because they gave several different-sized bands in the PCR reaction to clone as different DNA fragments.

Amplification of Peptide Antibiotics Synthetase Gene from Wild-type *Bacillus subtilis* Strain

In order to determine whether these PCR primers amplified the desired products, namely, biosynthetic gene fragments for peptide antibiotics, we determined the nucleotide sequences of major PCR products (bands 3 and 5 in lane 1, Fig. 2a) from the PCR reaction using *Bacillus subtilis* wild-type PB2 strain as positive control. The nucleotide sequence analysis of band 3 (designated as PB2-3) showed that the amplified product is a part of the surfactin synthetase gene (*srfAA*) from amino acid 614 to amino acid 712 (data not shown), proving that primers 1 and 2 amplified the desired products. We identified that the amplified product contained the

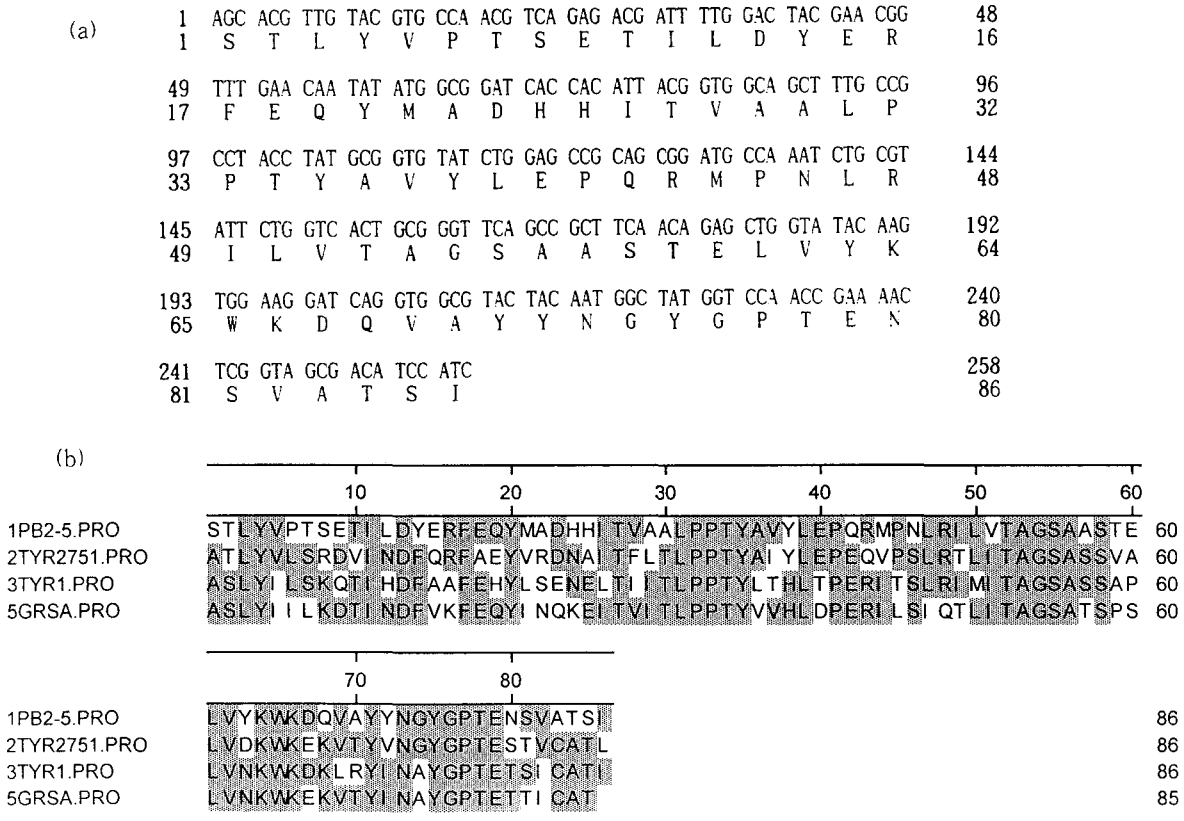

Fig. 3. Nucleotide sequence of one of the PCR products from *Bacillus subtilis* PB2, predicted amino acid sequence, and alignment with peptide synthetase genes.

(a) Nucleotide sequence and their predicted amino acid sequence of the major PCR product (band 5, Fig. 2a) from *Bacillus subtilis* PB2. (b) Alignment with various peptide synthetase genes using CLUSTAL V [8]. 1PB2-5 is the predicted amino acid sequence based on the nucleotide sequence of PCR product, band 5. 2TYR2751 and 3TYR1 are the amino acid sequences of two different amino acid activating domains of the tyrocidine synthetase gene and 5GRSA is that of the gramicidine synthetase gene. Identical amino acids are shaded.

conserved Core 2 region by nucleotide sequencing the PCR product and by alignment with the amino acid sequence of the surfactin synthetase gene.

The predicted amino acid sequence based on the DNA nucleotide sequence of the other major product (band 5, designated as PB2-5, Fig. 2a) showed significant homology to the sequences of various peptide antibiotics synthetase genes (Fig. 3a, b) including the tyrocidine synthetase gene of *Brevibacillus brevis* [(identity = 53% (46/86)] and gramicidine S synthetase gene of *Bacillus brevis* [(identity = 485 (41/85)]. However, it was found to be different from the nucleotide sequence of the surfactin

(a)

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  1  GGT ACA ACC GGT AAG CCA AAA GGG GTT ATG ATT GAG CAA GGC AGT GTC   48
  1   G   T   T   G   K   P   K   G   V   M   I   E   Q   G   S   V   16

 49  GGC AAT CTG GTA GAT GCT CTG TAT GAG CGA GTA TAC AGT CGG TAT GAT   96
 17   G   N   L   V   D   A   L   Y   E   R   V   Y   S   R   Y   D   32

 97  CAG CCG CTT CAC ATC GCA TGG CTG TCC GAA TTT GTA TTT GAC GCT TCG  144
 33   Q   P   L   H   I   A   W   L   S   E   F   V   F   D   A   S   48

145  GTG AAG CAA ATC TTT GCC AGC TTG CTG CTA GGT CAT ACC CTG CAT GTG  192
 49   V   K   Q   I   F   A   S   L   L   L   G   H   T   L   H   V   64

193  CGT GTC CGG TAC GTA CGC CCT GAG CGG AGA GCA TTG ATC GCC TAC TAC  240
 65   R   V   R   Y   V   R   P   E   R   R   A   L   I   A   Y   Y   80

241  CGC ACG CAC CGG ATT GAT CTG TCG GAC GGC ACA CCA GCG CAT CTG CAT  288
 81   R   T   H   R   I   D   L   S   D   G   T   P   A   H   L   H   96

289  ATT TTG AAC GAA AGC GTC AGC GTG ACA GAA GCG CCG GAT GTG AAG CAT  336
 97   I   L   N   E   S   V   S   V   T   E   A   P   D   V   K   H  112

337  TAC CTG ATC GGG GGC GAA GCC TTA TC                              362
113   Y   L   I   G   G   E   A   L                                  120
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(b)

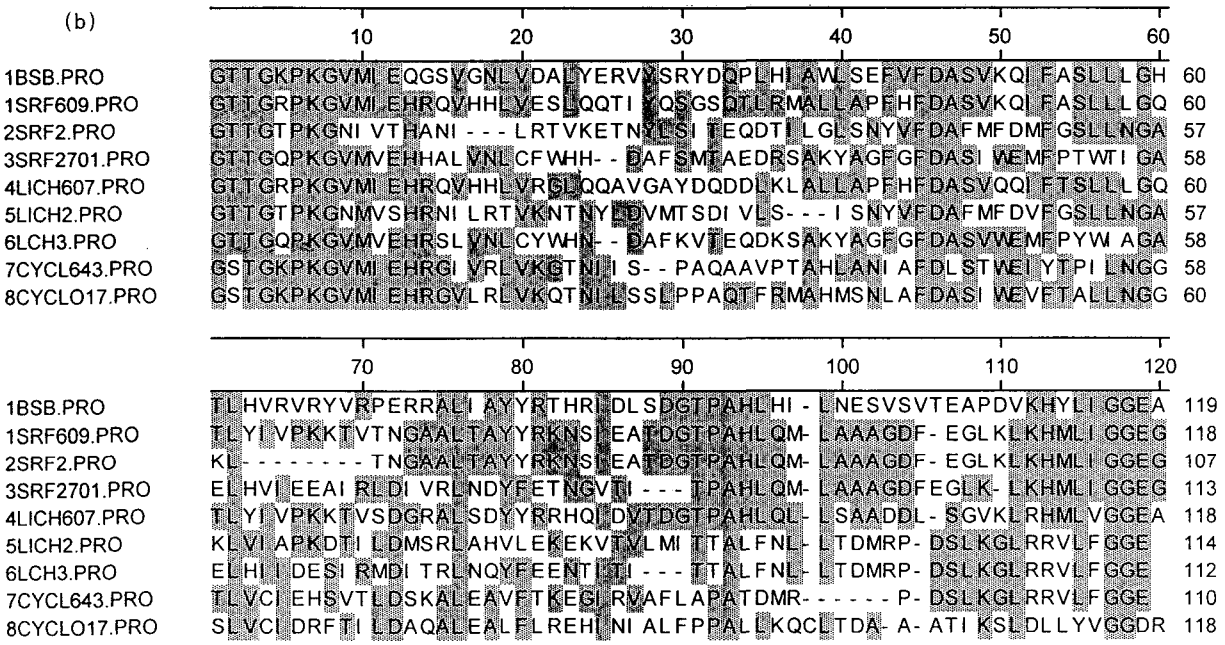

Fig. 4. Nucleotide sequence of PCR product from *Bacillus subtilis* 713, predicted amino acid sequence, and alignment with peptide synthetase genes.

(a) Nucleotide sequence and their predicted amino acid sequence of the major PCR product from *Bacillus subtilis* 713. (b) Alignment with various peptide synthetase genes using CLUSTAL V [8]. 1BSB is the predicted amino acid based on the nucleotide sequence of the PCR product from *Bacillus subtilis* 713. 1SRF609, 2SRF2, and 3SRF2701 are the amino acid sequence of three different domains of the surfactin synthetase gene and 4LICH607, 5LICH2, and 6LICH3 are the amino acid sequences of three different domains of the lichenysin synthetase gene. 7CYCL643 and 8CYCLO17 are the amino acid sequences of two different domains of the cyclosporin synthetase gene. Identical amino acids are shaded.

(a)

1	AAA	GGT	GTG	GTG	GTG	GAG	CAC	CGT	TCG	CTG	GGT	GCC	TAT	CTG	TTG	CGG	48
1	K	G	V	V	V	E	H	R	S	L	G	A	Y	L	L	R	16
49	AAC	CGG	GCG	ACG	TAC	GCG	GGG	GTT	GGT	GGA	GCG	TCT	CTG	CTG	CAT	ACG	96
17	N	R	A	T	Y	A	G	V	G	G	A	S	L	L	H	T	32
97	TCG	TTG	TCC	TTC	GAT	CTG	ACG	GTG	ACG	GCT	TTG	TTC	ACG	CCG	TTG	GTG	144
33	S	L	S	F	D	L	T	V	T	A	L	F	T	P	L	V	48
145	AGC	GGT	GGC	CGG	GTG	TGT	GTG	GGT	GAG	TTG	GAG	GAT	GGT	GCT	GGA	GTG	192
49	S	G	G	R	V	C	V	G	E	L	E	D	G	A	G	V	64
193	ACT	TTG	CTG	AAG	GTC	ACT	CCG	AGT	CAT	TTG	GCG	TTG	GTG	GAG	GGC	GAG	240
65	T	L	L	K	V	T	P	S	H	L	A	L	V	E	G	E	80
241	TGT	GAC	GTG	GTT	GCT	CCG	GCG	GGG	ACG	TTG	GTG	GTC	GGG	GGT	GAG	GTG	288
81	C	D	V	V	A	P	A	G	T	L	V	V	G	G	E	V	96
289	CTG	AGG	GGT	GAG	GTG	CTG	GAT	CGC	TGG	CGT	CGG	CGT	CAC	CCT	GAT	GTG	336
97	L	R	G	E	V	L	D	R	W	R	R	R	H	P	D	V	112
337	ACG	GTC	TTC	AAC	GCT	TAT	GGT	CC									359
113	T	V	F	N	A	Y	G										119

(b)

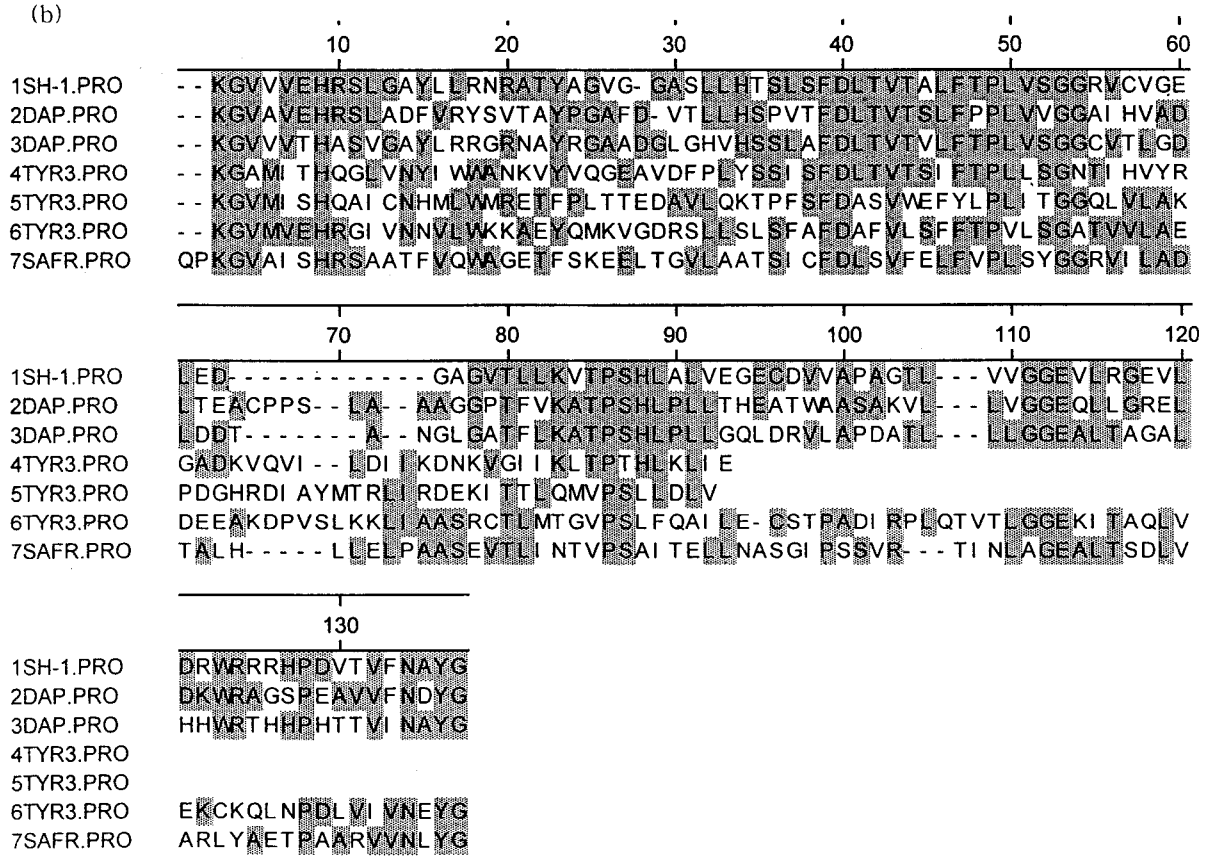

Fig. 5. Nucleotide sequence of PCR product from *Streptomyces hygroscopicus* KCTC 9169, their predicted amino acid sequence, and alignment with peptide synthetase genes.

(a) Nucleotide sequence and their predicted amino acid sequence of the major PCR product from *Streptomyces hygroscopicus* KCTC 9169. (b) Alignment with various peptide synthetase genes using CLUSTAL V [8]. 1SH-1 is the predicted amino acid based on the nucleotide sequence of the PCR product from *Streptomyces hygroscopicus* KCTC9169. 2DAP and 3DAP are the amino acid sequence of two different domains of the daptomysin synthetase gene. 4TYR3, 5TYR3, and 6YTR3 are the amino acid sequence of three different domains of the tyrocidine synthetase gene. 7SAFR is the the amino acid sequence of an amino acid activating domain of the saframycin synthetase gene. Identical amino acids are shaded.

synthetase gene of *Bacillus subtilis*. Based on the deduced amino acid sequences of homologous genes, we could locate the amplified DNA fragment (PB2-5) in the region between Core 2 and Core 3. Since the nucleotide sequence of the surfactin synthetase gene of *Bacillus subtilis* was reported, and the amplified product is not matched to the surfactin synthetase gene, the amplified product is likely a part of a peptide antibiotic synthetase gene other than the surfactin synthetase gene.

Amplification of Peptide Antibiotics Synthetase Gene from *Bacillus subtilis* 713 Isolated from Korean Soil

After confirming that the primers used for PCR could amplify the desired products, we decided to isolate peptide antibiotic synthetase genes from the strains which produce antifungal peptide antibiotics. Chromosomal DNA of *Bacillus subtilis* 713 isolated from Korean soil was used for the PCR reaction [11, 12, 22]. We obtained several PCR products using chromosomal DNA from this strain as a template with primers 1 and 2 (lane 1, Fig. 2b) and 2 bands with primers 3 and 4 (lane 4, Fig 2b). We isolated the 450 bp-long major band from the gel and determined the nucleotide sequence of the DNA fragment obtained by PCR (Fig. 4a). The predicted amino acid sequence based on the nucleotide sequence of the isolated DNA fragment showed high homology to various peptide antibiotics synthetase genes (Fig. 4b), including surfactin synthetase (aa 609 – aa 727) of *Bacillus subtilis* with an identity of 52% (62/120), and lichenysin synthetase of *Bacillus licheniformis* (aa 607 – aa 725) with identity of 48% (57/120). This suggests that the amplified product could be a part of a peptide synthetase gene for antifungal peptides.

Gu and coworkers also used PCR primers described in this study to amplify DNA fragments of biosynthetic genes using chromosomal DNA of the *Bacillus subtilis* species, isolated from Korean soil, which is also known to produce an antifungal peptide antibiotic (Gu *et al.*, Agricultural Science and Technology Institute, RDA, Korea, personal communication).

Amplification of Peptide Antibiotic Synthetase Gene from Actinomycetes

We also tested whether this technique could be used for the isolation of peptide synthetase genes from Actinomycetes. We used *Streptomyces hygroscopicus* KCTC 9169 because this strain is known to produce many secondary metabolites [31]. In addition, a preliminary experiment (data not shown) suggested that one of the secondary metabolites might be a peptide. When the chromosomal DNA of *S. hygroscopicus* KCTC 9169 was used for PCR reaction, several bands with the primer set of primers 1 and 2 (lane 2 in Fig. 2b) and one band with primers 3 and 4 (lane 5 in Fig. 2b) were obtained. We cloned the major band of the PCR products from *S. hygroscopicus* KCTC 9169 (lane 2 in Fig. 2b). The nucleotide sequence analysis of the PCR product showed that the amplified product was the DNA fragment encoding a peptide antibiotic synthetase gene based on its predicted amino acid sequence (Fig. 5a, b). The predicted amino acids of the PCR product showed an identity of 54% (72/134) to the daptomysin synthetase gene of *Streptomyces roseosporus*.

In conclusion, we were able to amplify DNA fragments which have significant homology to peptide synthetase genes with the PCR primers we designed. However, we cannot definitely conclude that these are the DNA fragments of the peptide synthetase genes for the antifungal compounds which the tested bacterial strains produce. This should be proved by further isolation of the rest of the genes and their functional analysis. However, this rapid and efficient technique using PCR can be used for the isolation of peptide antibiotics synthetase genes from various bacterial strains. Further cloning of peptide synthetase genes can be the foundation for the development of noble antibiotics by gene manipulation such as switching, deleting, and adding foreign amino acid-activating domains from other peptide synthetase genes as reported recently [29].

Acknowledgments

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