Identification and Cloning of a Gene Locus Encoding Peptide Synthetase of *Pseudomonas fluorescens* by Two Sets of PCR Primers

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A chromosomal locus encoding biosynthetic genes for a putative peptide synthetase of *Pseudomonas fluorescens* was identified and cloned. To achieve this, two sets of degenerated oligonucleotide primers KAGGA:SGTTG and TGD:LGG were used in PCR. These primers were selected based on highly conserved units of known peptide synthetases involved in adenylation and thiolation regions of *Bacillus subtilis*. The discrete amplified bands from PCR ca. 300 bp for KAGGA:SGTTG and ca. 500 bp for TGD:LGG proved to be integral part of the genomic DNA of *P. fluorescens* were cloned and sequenced. Sequence alignments of both fragments confirmed the putative peptide synthetase genes in *P. fluorescens*. The present study describes the identification and cloning of peptide synthetase genes of *P. fluorescens*, which can be used to identify a genetic locus encoding peptide synthetase in other microbial species.

Introduction

Peptide synthetases are large multienzyme complexes that catalyze the non-ribosomal synthesis of many bioactive products such as antibiotics. Enzymatically synthesized peptides are composed of peptide chains that may be modified by acylation or glycosylation. Most of them are derived from Bacilli and filamentous fungi (Stachelhaus and Marahiel, 1995). The pseudomonad also contributes many such special metabolites using non-ribosomal biosynthetic mechanism of the peptide synthetase (Rajendran *et al.*, 1996a). The well known models are phaseolotoxin from *P. syringae*, var. *phaseolica*, syringotoxin from *P. syringae,* the siderophores enterobactin and pyoverdine from *P. aeruginosa,* antibiotics pyrolnitrin, pyoluteorin, sperabillin, pyoverdine, azomycin, obafluorin, ferribactin and surfactin from *P. fluorescens* (Budzikiewicz, 1993).

This investigation was designed to identify and clone the chromosomal locus encoding a part of peptide synthetase of *Pseudomonas fluorescens* by PCR using two sets of degenerated oligonucleotide primers derived from conserved core sequences found only in the peptide synthetases.

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Materials and Methods

Pseudomonas fluorescens AU63, was cultured on nutrient broth at 30 °C (Rajendran *et al.*, 1998). The oligonucleotide sequences of four degenerated primers are given in Fig. 1. The PCR reaction mixture (each 100 µl) contained 1.5 µl of each of two primers (35 pM μl^{-1}), 10 µl of 10x buffer, 5 µl of dimethylsulfoxide, 10 µl of template DNA (0.1 ng μl^{-1}), 10 µl of dNTPs (concentration of each nucleotide: 2 mM), 61 µl of d:H_2O, and 1 µl of (1 U μl^{-1}) Taq DNA polymerase. A PCR run comprised 35 cycles. The denaturing, extension and annealing temperatures were kept at 94 °C, 72 °C and 50 °C, respectively for 1 minute each.

Plasmid preparation and PCR product purification were performed by using the Qiagen plasmid and QIA quick-spin PCR purification kit (Qiagen, USA). Cloning was carried out with a pCR-Script SK^+ cloning kit (Stratagene, USA). Standard protocols (Sambrook *et al.,* 1989) were followed for DNA preparation and manipulations. A non-radioactive chemiluminescence detection kit was used for Southern hybridization (ECL-Amers-

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ham). DNA sequence was performed by using automated DNA sequencer (ABI, USA). The sequence data were analyzed by DNAStrider and DNA Sequence Navigator computer programs. Sequence homology in the GenBank (Swissport) were searched by using the BLASTX program package of NCBI, USA.

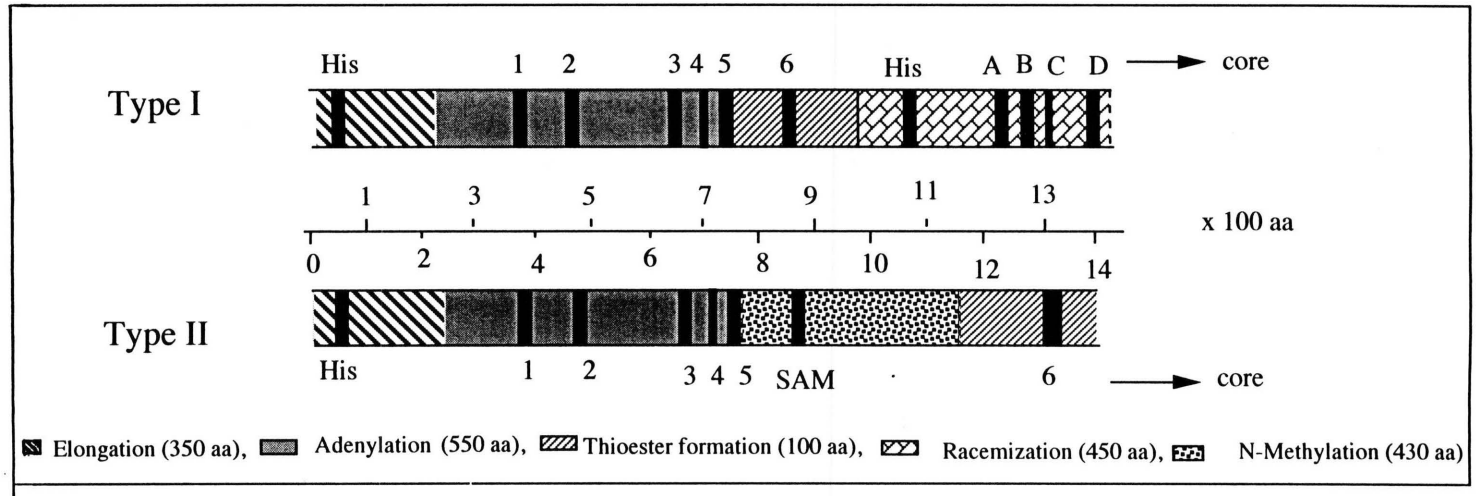
Results and Discussion

Peptide synthetases consist of many conserved motifs (Marahiel *et al.*, 1998) involved in substrate activation and thioester formation (Rajendran *et al.*, 1996a). Based on the conserved units, a method for identifying and cloning parts of putative peptide synthetase genes in *Pseudomonas fluorescens* was developed. According to this method, two sets of degenerated oligonucleotide primers (Fig. 1) derived from highly conserved adenylation and thiolation regions of *Bacillus subtilis* were used in PCR. The physical existence of discrete amplified bands in the genomic DNA (ca. 300 bp for KAGGA:SGTTG primers and ca. 500 bp for TGD:LGG primers) were identified by Southern hybridization (data not shown). The sequence analysis of the amplified fragment confirmed the relatedness to peptide synthetase. In order to identify larger restriction fragments containing more of the gene locus, the 500 bp fragment was used to probe the genomic DNA of *P. fluorescens*. The identified 4 kb *PstI* fragment was cloned in pBluescript II SK+ vector.

Comparison of the terminal sequence analysis of both 5′ and 3′ ends of 4 kb *PstI* clone with published sequence data confirmed the cloned fragment as a genuine part of a peptide synthetase. The presence of core sequences such as KIRGXRIEL, LP(N)YM(C)P and NGK were examined, when the second set of primers was used to sequence the fragment. The cores TGD and LGGHS were identified when universal and reverse primers were used to sequence both 5′ and 3′ termini of the clone. The computer alignments of the derived amino acid sequence with known peptide sequences have revealed the peptide synthetase homology as shown in Fig. 2. The biological role of the putative gene product is not known. However from achieving the amplification of peptide fragments using known peptide primers by PCR as well as the homologous identity from sequence analysis, it is surmised that as a peptide product. The earlier structural analysis (Katayama *et al.*, 1992) was established that *P. fluorescens* YK-437 does synthesis a pseudo peptide, sperabillin. However it can not be excluded that the sequence from the present study also involved in the biosynthesis of siderophore synthetase.

The feasibility of KAGGA and SGTTG primers for identifying peptide synthetase from a gene library of *Bacillus subtilis* without using PCR, was demonstrated earlier (Borchert *et al.*, 1992). Instead of gene library, a PCR based method was developed and established the feasibility of using this set of primers to identify and clone a part of putative peptide synthetase of *P. fluorescens* (Rajendran and Marahiel, 1996b). This was examined in *Bacillus brevis* and *B. subtilis* and observed the PCR amplification (unpublished data). A second set of primers (TGD and LGG) was used in *P. syringae* (Turgay and Marahiel, 1994). The present study includes both set of primers and established that the first set of primers (KAGGA and SGTTG) amplifies a 300 bp fragment and the second set of primers (TGD and LGG) amplifies a 500 bp of another fragment corresponding to peptide synthetase in *P. fluorescens*. The other oligonucleotide combinations (Marahiel *et al.*, 1997) such as SGTTGXPKG : TGD or KIRGXRIEL : LGGHS, or TGD : NGK were also experimented and confirmed no amplification of fragments in *P. fluorescens*.

Taken together, these results indicate that either one or both set of core sequences corresponding to peptide synthetases, exist in some microbial species. Hence, checking for the presence of the peptide synthetase gene is more useful when both sets of primers are used subsequently. If one set of primers fail to amplify a fragment due to unknown reasons, the other set of primers may support the amplification. The peptide synthetase identity can be additionally confirmed by checking *EcoRI* site of KAGGA and the amplified fragment can be cloned at same site, if the first set of primers is used. The presence of core sequences such as KIRGXRIEL, LP(N)YM(C)P, and NGK can confirm the identify if the second set of primers is used. The existence of amplified fragments in genomic DNA can be confirmed twice by Southern hybridization and twice by the sequence analysis, if two different fragments were obtained by PCR



Revised nomenclature for A & T cores and the consensus sequence*

Adenylation:
A1.....= L(TS)YxEL
A2 (Core 1) = LKAGxAYL(VL)P(Li)D
A3 (Core 2)= LAYxxYTSG(ST)TGxPKG
A4..... = FDxS
A5..... = NxYGPTE
A6 (Core3) = GELxIxGxG(VL)ARGYL
A7 (Core4) = Y(RK)TGDL
A8 (Core5) = GRxDxQVKIRGxRIELGEIE
A9.....= LPxYM(IV)P
A10.....= NGK(VL)DR
Thiolation:
T (Core 6) = DxFFxxLGG(HD)S(LI)

* Adapted from M.A.Marahiel *et al*, 1997

The degenerated oligonucleotides, derived from four core sequences:

KAGGA : 5′ - GGAATTCCTCAAGGCGGGCGGIGCCTACGTGCCCIACCC - 3′
G C TA G G

SGTTG : 5′ - GGAATTCCCTTGGGCIGGCCGGICGICCCGGAGGTGTAGA - 3′
C T C G G

TGD : 5′ - TACCGIACIGGIGATCTIGTICG - 3′
T T CTG G

LGG : 5′ - AT IGAGTCICCICCIGGGIAAAAGAA - 3′
AA AGG AAC CG A

Fig. 1. The biosynthetic process of the peptide synthetases and the specification of its sequence is brought about by the protein template that contains the appropriate number and correct order of activating units. Two types of amino acid activating modules [2], in peptide synthetases are shown here. This figure depicts the relative location, extension (size in amino acid residues), and organization of specialized modules such as elongation, adenylation, thioester formation, racemization, and N-methylation. The core 1 to 5 are the sequences involved in acyladenylation, and 6 is a conserved core motif of modules required for thioester formation [1]. N-methylation (VLE/DxGxGxG), peptide elongation (His, HHILxDGW), and optional racemization (His,HHILxDGW, A,AYxTExNDILLTAxG, B,EGHGRExIIE, C,RTVGWFTS MYPxxLD; and D,FNYLGQFD) are also shown. The revised nomenclature for adenylation and thiolation are given in a separate box. The degenerated oligonucleotide sequences of the two pairs of primers used in PCR are given separately.

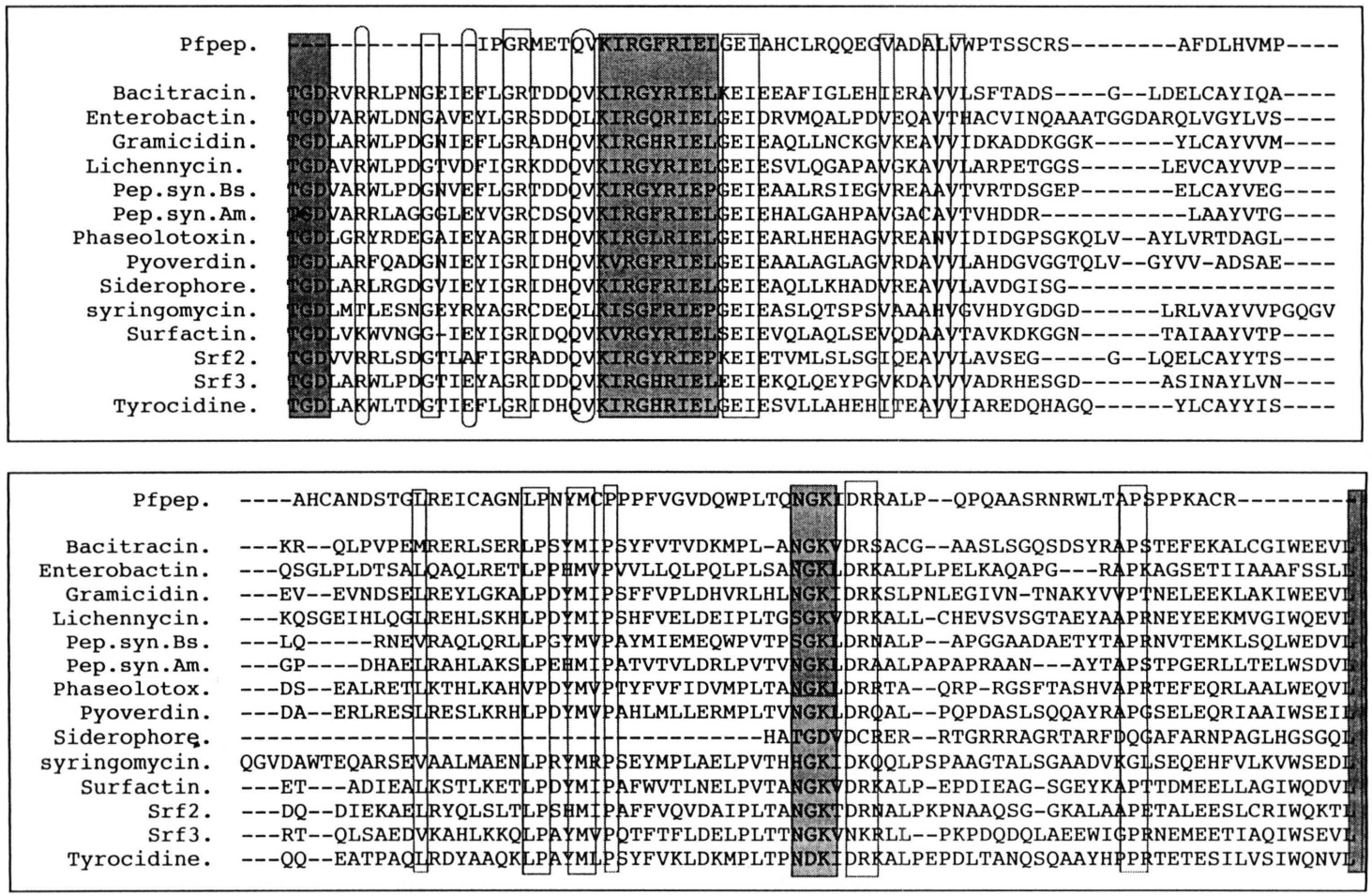


Fig. 2. Alignment of the derived amino acids sequence of the peptide synthetase fragment from *Pseudomonas fluorescens* (pfpep) with homologous fragments of various peptide synthetase genes. The sequence alignment was made using GenBank (Swissport) of BLASTX program of NCBI, USA. The gray shades indicate highly conserved core regions, white strips indicate high homology and blunt white strips indicate the possible homology.

as in the case of *P. fluorescens*. In conclusion, the two sets of primers usage is proved to be an authentic tool for *P. fluorescens* and it can be applied in many bacteria and fungi which are (un)known producers of peptide synthetases, to identify and clone a gene locus encoding putative peptide synthetases.

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