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Detection of Novel Polyketide Synthase Genes in *Sorangium cellulosum* Isolated in Korea

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DNA fragments encoding the ketosynthase (KS) domain of polyketide synthase (PKS) genes were amplified using polymerase chain reaction (PCR) from 9 strains of *Sorangium cellulosum* isolated in Korea, cloned into a plasmid vector and sequenced. A total of 83 cloned DNA fragments were analyzed, and similar fragments were excluded, leaving 43 independent DNA fragments encoding the KS domains. The predicted amino acid sequences of 32 fragments were 70%-100% identical to the amino acid sequences of already known PKS genes, while the remaining 11 fragments were ≤67% or less identical to the known sequences, suggesting that these genes are novel PKS genes.

Key words: Myxobacteria, polyketide synthase, PKS, *Sorangium cellulosum*

Introduction

Myxobacteria, gram-negative soil bacteria, are a rich source of bioactive secondary metabolites. More than 500 bioactive substances have been identified from myxobacteria [6, 12, 25]. The majority of the bioactive substances isolated from myxobacteria are polyketides or hybrids of polyketide and non-ribosomal peptides [25]. Polyketides biosynthesized from acyl-coenzyme A monomers by polyketide synthases (PKSs) [24] and non-ribosomal peptides biosynthesized from amino acids by non-ribosomal peptide synthetases [3] are two large classes of natural products that have important pharmaceutical properties. PKSs are composed of multiple covalently linked domains grouped into modules, each of which is responsible for a round of polyketide chain extension and functional group modification [2, 23]. Thus, PKSs provide an attractive framework for engineering assemblies that produce novel polyketides.

Among myxobacteria, *Sorangium cellulosum* is the most proficient producer of bioactive substances [5, 6]. Approximately 47% of the bioactive substances identified from myxobacteria are from this species [6], including epothilones– a potential new class of anticancer compounds. Because of their pharmaceutical importance, several PKS genes have been cloned from *S. cellulosum* and other species of myxobacteria [1]. However, genomic studies have indicated that there are still many other unidentified PKS genes in myxobacteria; these genes may produce novel polyketides [19, 22, 25]. The detection of PKS genes by polymerase chain reaction (PCR) without cloning has also indicated the presence of many unidentified PKS genes in myxobacteria [13, 15]. Using PCR, Li *et al*. detected 56 ketosynthase (KS) domains of PKSs from 10 *S. cellulosum* strains that appeared to be novel; these genes had identities between 54% and 83% to known PKSs in the databases [15]. Komaki *et al*. detected 80 PKS genes by PCR from diverse myxobacteria that appeared to be novel with less than 70% identity to known PKSs [13].

Previously, we reported the isolation of 591 *S. cellulosum* strains from soil samples collected from various locations in Korea [7]. In this paper, we report the detection of PKS genes from 9 strains of *S. cellulosum,* each of which were isolated from a different province of Korea.

Materials and Methods

Strains and Culture Conditions

All strains of *S. cellulosum* used in this study were isolated in Korea (Table 1) [7]. Competent cells of *Escherichia coli* DH5α were purchased from Donginbiotech Co., Korea. ST21P medium [7] was used to culture *S. cellulosum,* and LB medium [20] was used to grow *E. coli*.

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Table 1. *Sorangium cellulosum* strains used in this study.

Strains	Geographical origin	References
KYC3013	Jeju, Jeju-do	8
KYC3014	Haenam, Jeollanam-do	7
KYC3043	Dangjin, Chungcheongnam-do	7
KYC3046	Icheon, Gyeonggi-do	7
KYC3048	Yeongdeok, Gyeongsangbuk-do	7
KYC3060	Jumunjin, Gangwon-do	7
KYC3074	Danyang, Chungcheongbuk-do	7
KYC3175	Muju, Jeollabuk-do	7
KYC3176	Changnyeong, Gyeongsangnam-do	7

S. cellulosum was cultured at 32°C and *E. coli* was cultured at 37°C.

Isolation of Genomic DNA from *S. cellulosum*

Cells grown on ST21P plates for 7 days were dissolved in 15 mL lysis solution (20 mM Tris-HCl (pH 8.0), 10 mM NaCl, 0.5% SDS, and 100 μg/mL proteinase K) in a 50 mL tube and incubated at 50°C for 12 hours. The lysate was extracted with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) mixture and then with 1 volume of chloroform. The extraction mixture was centrifuged (5,000 rpm, 10°C for 10 min), and the supernatant was transferred to a new tube. Next, a 0.1 volume of 3 M sodium acetate solution (pH 5.5) and two volumes of 100% ethanol were added to the solution to precipitate the genomic DNA. The precipitated DNA was washed with ice-cold 70% (v/v) ethanol, dried, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Polymerase Chain Reaction and Sequence Analysis

The PKS genes of *S. cellulosum* were amplified using the genomic DNA isolated from *S. cellulosum* strains and a set of oligonucleotides (5'-GCSATGGAYCCSCARCAR-CGSVT-3' and 5'-GTSCCSGTSCCRTGSSCYTCSAC-3' reported by Schirmer *et al*. [21]) as primers. Denaturation was conducted at 94°C for 30 sec, annealing at 66°C for 30 sec, and extension was carried out at 72°C for 1 min. A total of 25 cycles of PCR were performed with *Taq* DNA polymerase (Solgent Co., Korea). The resultant PCR fragments were ligated into a pGEM-T Easy plasmid vector (Promega, USA), and the ligation products were used to transform *E. coli* DH5α, resulting in the cloning of the PCR fragments into the plasmid vector. The DNA sequences of the cloned PCR fragments were determined by Macrogen Inc., Korea, using SP6 and T7 primers. The DNA sequences and the predicted amino acid sequences were analyzed using NCBI BLAST [9] and ClustalX2 [14] programs.

Results and Discussion

Detection of PKS Genes from *S. cellulosum* by PCR

S. cellulosum strains KYC3013, KYC3014, KYC3043, KYC3046, KYC3048, KYC3060, KYC3074, KYC3175, and KYC3176 were isolated from 9 different provinces of Korea [7]. To detect the PKS genes, PCR was carried out using the genomic DNA of these strains as templates and two oligonucleotides that bind specifically to the KS domains of PKS. PCR with the genomic DNA from all 9 strains yielded about 680 bp DNA fragments, which were the expected size of the PCR product. The resultant PCR fragments were then cloned into a plasmid vector, and the DNA sequences of the cloned fragments were determined. A total of 83 fragments encoding the KS domains of the PKS genes were analyzed, and similar fragments with more than 90% identity among the fragments from the same strain were excluded, which left 43 independent cloned PCR fragments.

PKS Genes Highly Homologous to Known PKS Genes

The predicted amino acid sequences of the cloned fragments were compared to the amino acid sequences in GenBank by use of the BLASTP program (Table 2). It appears that the predicted amino acid sequences of 32 fragments (of the 43 cloned fragments) were 70%-100% identical to those in GenBank. Among them, 18 fragments were 70%-100% identical to those encoded by PKS genes whose polyketide products were already known (Table 3). The predicted amino acid sequence of cloned fragment 3013-10, which was from the strain KYC3013, was 99% identical to SpiD (Fig. 1, Table 2). The amino acid sequence of fragments 3043-2, 3043-8, 3043-13, and 3043-15, which were from strain KYC3043, were 99% identical to SpiI, SpiE, SpiD, and SpiF, respectively (Fig. 1, Table 2). The deduced amino acid sequence of fragment 3175-2, which was from strain KYC3175, was 99% identical to SpiE (Fig. 1, Table 2). SpiI, SpiE, SpiD, and SpiF are enzymes involved in the biosynthesis of spirangiene [4]. Thus, these results indicate that strains KYC3013, KYC4043, and KYC3175 carry genes for the biosynthesis of spirangiene. The predicted amino acid sequences of cloned fragments

Table 2. Polyketide synthase genes detected by polymerase chain reaction (PCR) in this study.

No.	Cloned PCR fragment	Accession no.	Size[a] (bp)	The most similar protein sequences in GenBank			
				Accession no.[b]	Type[c]	Product	Identity (%)
1	3013-10	GU053740	634	CAL58681	cloned gene (*spiD*)	Spirangiene	99
2	3013-14	GU053741	637	BAG69089	PCR fragment	?	99
				AAF62884	cloned gene (*epoE*)	Epothilone	66
3	3013-15	GU053742	634	BAG69077	PCR fragment	?	99
				ABK32259	cloned gene (*ambE*)	Ambruticin	69
4	3013-19	GU053743	637	ABD17630	PCR fragment	?	92
				AAF62884	cloned gene (*epoE*)	Epothilone	65
5	3013-20	GU053744	634	BAG69090	PCR fragment	?	98
				ABK32259	cloned gene (*ambE*)	Ambruticin	71
6	3014-1	GU053745	634	BAG69048	PCR fragment	?	98
				AAF26921	cloned gene (*epoC*)	Epothilone	71
7	3014-2	GU053746	649	BAG69044	PCR fragment	?	99
				AAS98787	cloned gene (*jamP*)	Jamaicamide	58
8	3014-6	GU053747	637	BAG69041	PCR fragment	?	79
				CAQ18829	cloned gene (*ajuB*)	Ajudazol	63
9	3014-7	GU053748	637	BAG69041	PCR fragment	?	99
				CAQ18829	cloned gene (*ajuB*)	Ajudazol	66
10	3014-13	GU053749	634	BAG69046	PCR fragment	?	99
				CAI43932	cloned gene (*disA*)	Disorazol	83
11	3043-2	GU053750	628	CAD43450	cloned gene (*spiI*)	Spirangiene	99
12	3043-4	GU053751	637	AAF62883	cloned gene (*epoD*)	Epothilone	100
13	3043-8	GU053752	634	CAL58682	cloned gene (*spiE*)	Spirangiene	99
14	3043-13	GU053753	634	CAL58681	cloned gene (*spiD*)	Spirangiene	99
15	3043-15	GU053754	634	CAL58683	cloned gene (*spiF*)	Spirangiene	99
16	3043-16	GU053755	637	BAG69074	PCR fragment	?	98
				AAF62883	cloned gene (*epoD*)	Epothilone	70
17	3043-17	GU053756	637	AAF62884	cloned gene (*epoE*)	Epothilone	99
18	3046-1	GU053757	646	YP_001618911	genomic sequence	?	96
				AAS98784	cloned gene (*jamM*)	Jamaicamide	60
19	3046-7[d]	GU053758	637	BAG69074	PCR fragment	?	64
				CAJ46689	cloned gene (*cmdA*)	Chondramide	64
20	3046-10	GU053759	631	YP_001614779	genomic sequence	?	54
				CAI43932	cloned gene (*disA*)	Disorazol	51
21	3046-15	GU053760	631	YP_001614779	genomic sequence	?	53
				CAI43932	cloned gene (*disA*)	Disorazol	52
22	3046-17	GU053761	649	ABD17653	PCR fragment	?	96
				AAS98787	cloned gene (*jamP*)	Jamaicamide	57
23	3048-1	GU053763	637	BAG69041	PCR fragment	?	98
				CAQ18829	cloned gene (*ajuB*)	Ajudazol	66
24	3048-17	GU053764	637	ABD17668	PCR fragment	?	97
				AAK19883	cloned gene (*sorA*)	Soraphen	76
25	3048-20	GU053765	637	AAF62884	cloned gene (*epoE*)	Epothilone	67
26	3060-2	GU053766	649	ABD17655	PCR fragment	?	99
				AAS98784	cloned gene (*jamM*)	Jamaicamide	58
27	3060-7	GU053767	636	BAG69065	PCR fragment	?	98
				CAJ46689	cloned gene (*cmdA*)	Chondramide	67
28	3060-8	GU053768	633	ABD17674	PCR fragment	?	95
				CAI43932	cloned gene (*disA*)	Disorazol	58
29	3060-10	GU053769	634	ABK32287	cloned gene (*jerA*)	Jerangolid	96
30	3060-12	GU053770	631	CAD19088	cloned gene (*stiD*)	Stigmatellin	64

Table 2. Continued

No.	Cloned PCR fragment	Accession no.	Size[a] (bp)	The most similar protein sequences in GenBank			
				Accession no.[b]	Type[c]	Product	Identity (%)
31	3060-17	GU053771	634	ABK32259	cloned gene (*ambE*)	Ambruticin	96
32	3074-5	GU053772	638	AAF62883	cloned gene (*epoD*)	Epothilone	65
33	3074-7	GU053773	631	AAK57189	cloned gene (*mxaE*)	Myxalamid	78
34	3074-8	GU053774	637	ABD17671	PCR fragment	?	99
				AAK57190	cloned gene (*mxaF*)	Myxalamid	71
35	3074-11	GU053775	637	BAG69041	PCR fragment	?	99
				CAQ18829	cloned gene (*ajuB*)	Ajudazol	66
36	3074-16	GU053776	640	AAF62883	cloned gene (*epoD*)	Epothilone	64
37	3175-2	GU053777	634	CAL58682	cloned gene (*spiE*)	Spirangiene	99
38	3175-16	GU053778	637	ABD17630	PCR fragment	?	76
				AAS98783	cloned gene (*jamL*)	Jamaicamide	62
39	3176-7	GU053779	637	AAF62883	cloned gene (*epoD*)	Epothilone	77
40	3176-8	GU053780	640	AAT70105	cloned gene (*curJ*)	Curacin	67
41	3176-11	GU053781	640	CAD19092	cloned gene (*stiH*)	Stigmatellin	63
42	3176-14	GU053782	640	CAD19086	cloned gene (*stiB*)	Stigmatellin	59
43	3176-18	GU053783	637	CAJ46690	cloned gene (*cmdB*)	Chondramide	67

[a]The size of the cloned PCR fragments after the length of primers was subtracted.
[b]Accession numbers of the most similar protein sequences in GenBank are shown. If the most similar protein sequence in the databases was one deduced from the gene whose polyketide product was not known, the accession number of the most similar protein sequence originating from the gene with a known product is also shown.
[c]Cloned gene: complete amino acid sequence deduced from a cloned gene with known polyketide products; PCR fragment: partial amino acid sequence deduced from a PCR fragment of a gene whose polyketide products are not known; genomic sequence: complete amino acid sequence originated from genomic sequence.
[d]Cloned PCR fragments 67% or less identical to published sequences in the databases are underlined.

Table 3. Strains of *Sorangium cellulosum* and their polyketide synthase genes detected by polymerase chain reaction.

Strains	Number of cloned PCR fragments			
	Encoding polyketide synthases (PKSs)	After similar clones were excluded	70%–100% identical to known PKSs	67% or less identical to known PKSs
KYC3013	12	5	5(3)[a]	0
KYC3014	9	5	5(3)	0
KYC3043	9	7	7(1)	0
KYC3046	10	5	2(1)	3
KYC3048	9	3	2(1)	1
KYC3060	10	6	5(3)	1
KYC3074	7	5	3(1)	2
KYC3175	9	2	2(1)	0
KYC3176	8	5	1(0)	4
Total	83	43	32(14)	11

[a]( ): Number of cloned PCR fragments that were 70%-100% identical to PKSs whose polyketide products have not been identified but 69% or less identical to PKSs whose polyketide products have been identical.

3043-4 and 3043-17, which were from strain KYC3043, were 100% and 99% identical to EpoD and EpoE, respectively (Fig. 1, Table 2). EpoD and EpoE are enzymes involved in the biosynthesis of epothilone [10, 16]. The predicted amino acid sequence of cloned fragment 3060-10, which was from strain KYC3060, was 96% identical to JerA (Fig. 1, Table 2)–an enzyme responsible for the biosynthesis of jerangolid [11]. The amino acid sequence of cloned fragment 3060-17 was 96% identical to AmbE (Fig. 1, Table 2), which is an enzyme responsible for the biosynthesis of ambruticin [11]. Thus, these results indicate that strain KYC3043 carries genes for the biosynthesis of

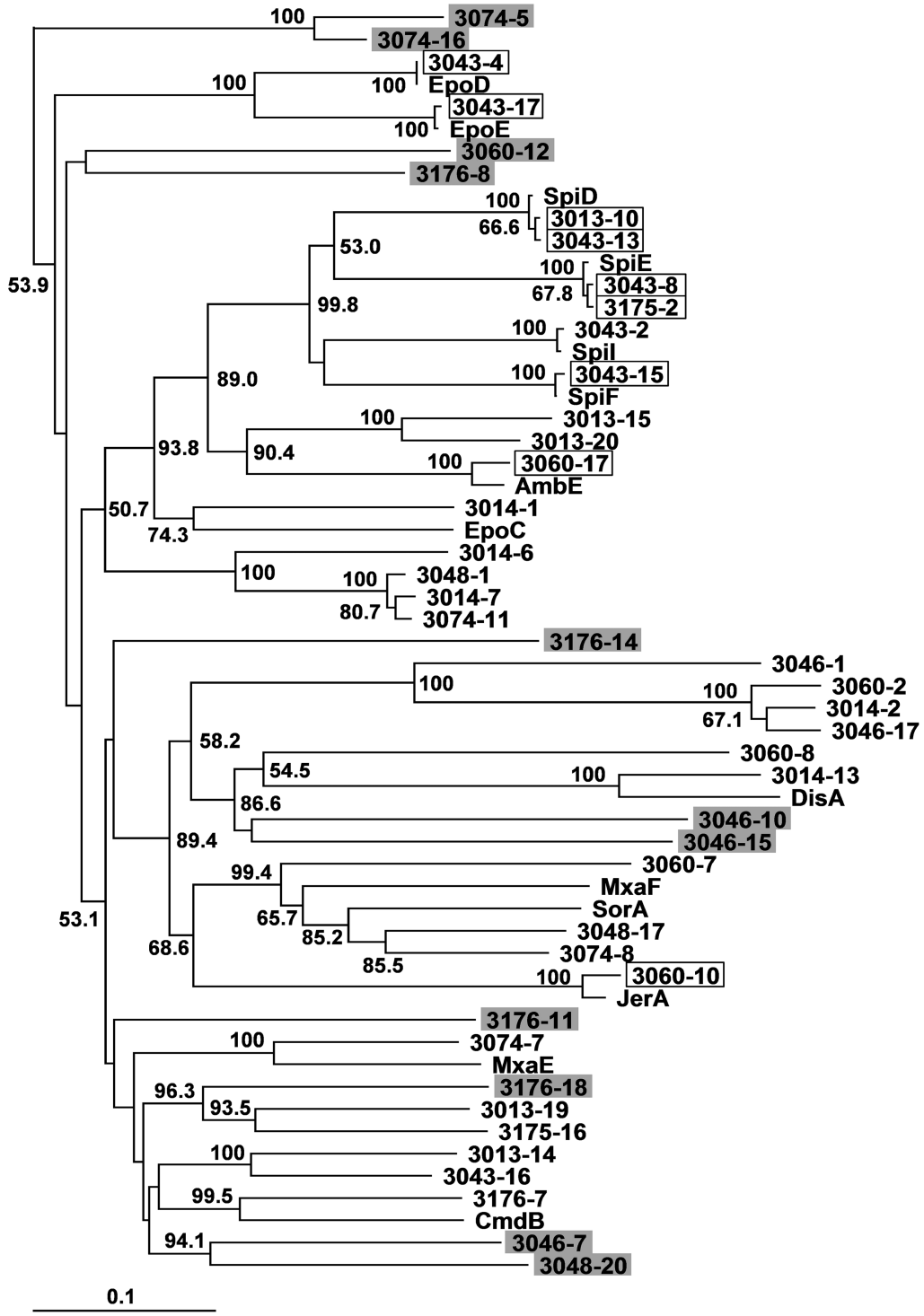

Fig. 1. Neighbor-joining tree based on the amino acid sequences of the KS domains in PKSs. Only the bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are indicated at the nodes. The 212 amino acid sequence (104–316 aa) of EpoA (AAF62880) was used as an outgroup (not shown). Cloned PCR fragments ≤67% identical to the reported amino acid sequences in the database are shaded and those 96%–100% identical to known PKS genes are boxed. Scale bar, 0.1 substitutions per nucleotide position.

epothilone and that KYC3060 carries genes for the biosynthesis of jerangolid and ambruticin.

Meanwhile, the deduced amino acid sequences of the other 14 fragments among the 32 cloned fragments were 71%–99% identical to those encoded by PKS genes whose polyketide products were not identified yet. They were

only 69% or less identical to PKSs with known polyketide products (Table 3). For instance, the deduced amino acid sequence of fragment 3013-14 was 99% identical to a PKS (BAG69089) whose product had not been identified, while it was 66% (to AAF62884) or less identical to PKSs whose polyketide products were known. BAG69089 is the deduced amino acid sequence of a partially PCR amplified PKS gene whose product is unknown [13], whereas AAF62884 is the complete amino acid sequence of the EpoE protein, a type I polyketide synthase containing epothilone synthase modules 7 and 8, involved in epothilone biosynthesis [10]. Since their products have not been identified yet and their sequence similarities to the KS genes with known products are low, it is expected that characterization of these genes would lead to the discovery of new polyketide compounds from *S. cellulosum*.

PKS Genes ≤69% Identical to Known PKS Genes

The remaining 11 other cloned PCR fragments of the 43 cloned fragments were ≤67% identical to sequences in the database (Table 3). Komaki *et al*. reported that KS domains involved in the synthesis of structurally related polyketide molecules in *Streptomyces* were, in almost all cases, more than 70% identical to each other [13]. Based on this, they classified the PKS genes whose sequences were less than 70% identical to the amino acid sequences of known genes as novel PKS genes. If this is true, many of the 11 cloned fragments that we have detected in this study would be novel PKS genes that synthesize novel PKS compounds. Characterization of these genes would lead to the cloning of new PKS genes or the discovery of new polyketide compounds.

Strain KYC3013 is known to have an epothilone biosynthetic gene cluster and produce epothilone [8]. However, none of epothilone biosynthetic genes were detected in KYC3013 (Table 2), indicating that the PCR-based detection method used in this study was not sensitive enough to detect all PKS genes in the strains. We reason that if the primers bind one PKS gene with higher affinity than the other PKS genes, the chance that the other genes would be amplified is low. As a result, genes with a low affinity to the primers would be difficult to detect by PCR and cloning.

Despite this problem, 11 fragments of the 43 cloned PCR fragments were ≤67% identical to published amino acid sequences in the database and are expected to carry novel PKS genes. Interestingly, 4 out of 5 cloned PCR fragments obtained from strain KYC3176 (3176-8, 3176-14, 3176-11, and 3176-18) were ≤67% identical to published amino acid sequences (Table 2). The other cloned fragment 3176-7 was 77% identical to EpoD. Because the EpoD epothilone synthases from different strains share more than 98% identity among their amino acid sequences, it is unlikely that strain 3176-7 encodes a PKS involved in epothilone biosynthesis. Thus, it appears that that all 5 cloned PCR fragments from KYC3176 carry novel PKS genes. In addition, 3 fragments from KYC3046, 2 fragments from KYC3074, and 1 fragment from KYC3048 and KYC3060 appear to carry novel PKS genes. *S. cellulosum* is known to carry multiple polyketide synthesis pathways [22], thus it is possible that each of these genes is involved in production of different polyketide compounds.

Currently, we are trying to knock out these genes to generate mutants that are unable to produce the polyketides biosynthesized by these genes. Characterization of these mutants will accelerate the discovery of new polyketide molecules and cloning of their biosynthetic genes.

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국문초록

국내에서 분리한 *Sorangium cellulosum*의 신규 Polyketide Synthase 유전자 검출

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국내에서 분리한 9균주의 *Sorangium cellulosum*로부터 중합효소연쇄반응(PCR)을 통해 polyketide synthase(PKS)의 ketosynthase(KS) domain을 암호화하는 DNA를 증폭하고, 플라스미드 벡터에 클로닝한 후, 염기서열을 결정하였다. 전체 83개의 클로닝된 DNA 조각을 분석하여 유사한 조각을 배제한 결과, 43조각이 KS domain을 암호화하는 독립된 DNA 조각으로 판명되었다. 43조각 중 32조각의 아미노산 서열이 이미 클로닝된 PKS 유전자의 아미노산 서열과 70%-100% 유사하였으며, 나머지 11 조각은 알려진 서열과 67% 이하의 상동성을 가져 새로운 PKS 유전자일 가능성이 매우 높음을 보여주었다.