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Cloning and Analysis of a Type II Polyketide Synthase Gene Cluster from *Streptomyces toxytricini* NRRL 15,443

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A standard type II polyketide synthase (PKS) gene cluster was isolated while attempting to clone the biosynthetic gene for lipstatin from *Streptomyces toxytricini* NRRL 15,443. This result was observed using a Southern blot of a *Pst*I-digested *S. toxytricini* chromosomal DNA library with a 444 bp amplified probe of a ketosynthase (KS) gene fragment. Four open reading frames [thioesterase (TE), β-ketoacyl systhase (KAS), chain length factor (CLF), and acyl carrier protein (ACP)], were identified through the nucleotide sequence determination and analysis of a 4.5 kb cloned DNA fragment. In order to confirm the involvement of a cloned gene in lipstatin biosynthesis, a gene disruption experiment for the KS gene was performed. However, the resulting gene disruptant did not show any significant difference in lipstatin production when compared to wild-type *S. toxytricini.* This result suggests that lipstatin may not be synthesized by a type II PKS.

Keywords: Type II polyketide synthase, *S. toxytricini*, lipstatin, β-ketoacyl synthase, chain length factor, acyl carrier protein, thioesterase

Lipstatin produced by *Streptomyces toxytricini* is a strong inhibitor of pancreatic lipase (Hochuli *et al.*, 1987; Weibel *et al.*, 1987). The reduced form of lipstatin, orlistat (tetrahydrolipstatin), is currently used to prevent obesity by interfering with the digestion and absorption of lipid compounds in the intestine (Zhi *et al.*, 1996; Hollander *et al.*, 1998).

Recently, the biosynthetic pathway for lipstatin has been revealed via a radioisotope feeding experiment (Eisenreich *et al.*, 1997; Goese *et al.*, 2000; Goese *et al.*, 2001; Schuhr *et al.*, 2002). According to previous reports, lipstatin is produced by Claisen condensation of two fatty acids, 3-hydroxytetradeca-5,8-dienoic acid and hexylmalonic acid, followed by the esterification of *N*-formylleucine on a polyketide backbone (Fig. 1). Similarly, the well-known hepatic 3-hydroxy-3-methylglutaryl (HMG) CoA reductase inhibitors used as anti-lipidemic agents, lovastatin (produced from *Aspergillus terreus*) and compactin (produced from *Penicillium citrinum*), are known to be synthesized via the esterification of monacolin L, which is produced from a nonaketide synthase, and 2-methylbutyric acid produced from a diketide synthase enzyme (Hendrickson *et al.*, 1999; Kennedy *et al.*, 1999; Abe *et al.*, 2002).

Despite the findings of isotope feeding experiments (Eisenreich *et al.*, 1997; Schuhr *et al.*, 2002), the gene cloning of a type II polyketide synthase (PKS) was attempted in order to isolate the biosynthetic gene cluster for lipstatin, under the assumption that hydroxytetradeca-5,8-dienoic acid and hexylmalonic acid for lipstatin backbone may be produced by a type II PKS, similarly to the polyketide synthesis of the HMG CoA reductase inhibitors. The involvement of the cloned gene in lipstatin biosynthesis was also investigated.

Materials and Methods

Bacterial strains and cultivation

A natural producer of lipstatin, *S. toxytricini* NRRL 15,443 was kindly provided by Northern Regional Research Laboratory (USA) and was cultivated on growth media (per l; starch 10 g, casamino acids 0.3 g, KNO_3 2 g, NaCl 2 g, K_2HPO_4 2 g, $MgSO_4{\cdot}7H_2O$ 50 mg, $CaCO_3$ 20 mg, $FeSO_4{\cdot}7H_2O$ 10 mg, pH 7.4) at 28°C for three days. The cloning hosts used were *E. coli* JM109 [*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F'(*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ M15)], *E. coli* DH5α [*recA1 supE44 hsdR17 endA1 gyrA96 thi-1 relA1* Δ*lacU169*(φ80*lacZ*ΔM15)], and *E.*

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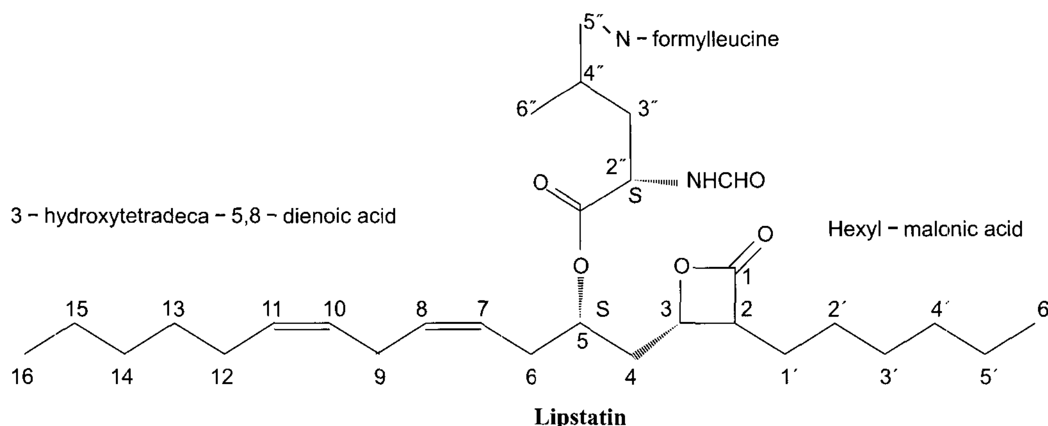

Fig. 1. The chemical structure of lipstatin. Lipstatin is composed of a polyketide backbone made from 3-hydroxytetradeca-5,8-dienoic acid and hexylmalonic acid, and *N*-formylleucine.

coli ET 12,567 [F$^-$ *dam-13*::Tn*9 dcm-6 hsdM hsdR recF143 zjj-202*::Tn*10 galK2 galT22 ara14 pacY1 xyl-5 leuB6 thi-1*]. Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0) was used to cultivate the *E. coli* strains. Ampicillin (50 μg/ml), 0.1 mM isoproryl-β-D-thiogalactopyranoside (IPTG), and 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were added to this medium when necessary.

Plasmid and DNA manipulation

The cloning vector, pGEM-T easy vector, was purchased from Promega Co. (USA) and the pGEM-3Zf(+) vector was purchased from Applied Biosystems (USA). The pKC1139 plasmid and pFDNEO-S plasmid were kindly supplied by Prof. Jae-Kyoung Sohng of Sunmoon University (Korea). The *E. coli* ET 12,567 harboring pKC1139 plasmid was maintained on LB medium containing 50 μg/ml apramycin and 20 μg/ml kanamycin. Plasmid manipulation, chromosomal DNA preparation, restriction digestion, DNA fragment isolation and cloning techniques were performed according to standard procedure (Kieser *et al.*, 2000; Sambrook and Russell, 2001).

Gene amplification

The KS gene was amplified from 2 μg/ml *S. toxytricini* chromosomal DNA via a polymerase chain reaction (PCR) with 1x *Taq* DNA polymerase buffer [50 mM Tris-HCl, pH 9.0, 15 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.1 mg/ml BSA, 0.5% DMSO], 0.5 pmol/μl primers, 0.1 mM dNTP, and 0.02 U/μl EF-*Taq* DNA polymerase in 25 μl reaction mixture, using the GeneAmp PCR system 2400 (Perkin-Elmer Co., USA). The F-1: 5'-C CGGTCGCCGAGGGCTTC-3', F-2: 5'-GCCCGGGA GGCGTTCGCC-3', F-3: 5'-GTCTCCACCGGCTGCA CC-3', and R-1: 5'-GCCGGAGCCGTGCGCGTT-3' primers were designed from the known type II PKS genes. The time profile was initial denaturation for 2 min at 95°C, 25 cycles of 1 min denaturation at 94°C and 2.5 min of annealing and synthesis at 68°C, followed by final synthesis for 5 min at 68°C.

Screening of genomic library

The genomic library of *S. toxytricini* was constructed by ligating 4-5 kb segments of *Pst*I-cut chromosomal DNA into the pGEM-3Zf(+) vector. The genomic library was screened with the amplified KS gene fragment labeled with [α-^{32}P]-dCTP using a DecaLabel™ DNA labeling kit (MBI Fermentas, Lithuania). The colony hybridization was done following the standard protocol (Sambrook and Russell, 2001)

DNA sequencing and analysis

The nucleotide determination of a screened clone was entrusted to Genotech Co. Ltd. (Korea). The DNA sequence was analyzed using FramePlot version 2.3.2 (Ishikawa and Hotta, 1999). DNA and deduced protein sequence homology searches of databases were performed using the BLAST program (Altschul *et al.*, 1990). Multiple alignment was performed using the ClustalW program (Thompson *et al.*, 1994).

Gene disruption

In order to confirm the involvement of the cloned PKS gene in lipstatin biosynthesis, disruption of the β-ketoacyl-ACP synthase (KAS) gene was attempted by homologous recombination using the pKC1139 plasmid (Bierman *et al.*, 1992; Yong and Byeon, 2005). Thus, a 935 bp DNA region of the neomycin resistance gene (*neo*^{R}) was amplified from the pFDNEO-S plasmid (Dennis and Brzezinski, 1991) using two primers; neo-F: 5'-GCTTCATGACCCAGAATCGG-3', which contained a *Bsp*HI site, and neo-R: 5'-CTCTAGGGT

CGGATCCCCAT-3', which contained a *Bam*HI site. The amplified *neo*^{R} gene was inserted using *Nco*I and the *Bgl*II-cut pPKSII plasmid, which is located in the middle region of the KS gene. The construct of the KS gene, which contained the *neo*^{R} gene, was ligated into the *Hin*dIII and *Eco*RI sites of pKC1139 to give pKC-KS, the disruption cassette for the insertional inactivation of the KS gene. Finally, the pKC-KS plasmid was electrotransformed into *E. coli* ET 12,567/pUZ8002 and conjugal transfer to *S. toxytricini* NRRL 15,443 was carried out with slight modification of the established method (Kieser *et al.,* 2000). The disruptant resistant to neomycin but sensitive to apramycin was obtained following several generations of culture at 28°C, 37°C and then at 28°C in R2YE medium containing neomycin (20 μg/ml) or apramycin (50 μg/ml) with nalidixic acid (50 μg/ml).

Determination of lipstatin amount

The parent strain of *S. toxytricini* and its gene disruptant were cultivated on growth media at 28°C for three days. The 2% seed cultures were inoculated into production media (2% tryptic soy broth and 2% lactose) and fermented at 28°C for three days. The culture broth after centrifugation was extracted with 3 volumes of ethyl acetate, and was dried *in vacuo*. After dissolving with 1 ml ethanol, the amount of lipstatin was determined while the degree of inhibition of pancreatic lipase activity was monitored via fluorospectrometry using 4-methylumbelliferyl oleate as a substrate (Lim *et al.*, 2006).

Results

Cloning of type II PKS gene from S. toxytricini chromosomal DNA

Under the assumption that the lipstatin backbone is synthesized by a heptaketide synthase and a tetraketide synthase, as in the case of lovastatin or compactin, cloning of the type II PKS gene was attempted in order to isolate a lipstatin biosynthetic gene cluster. In order to successfully clone the type II PKS gene, the KS gene fragment was amplified from *S. toxytricini*

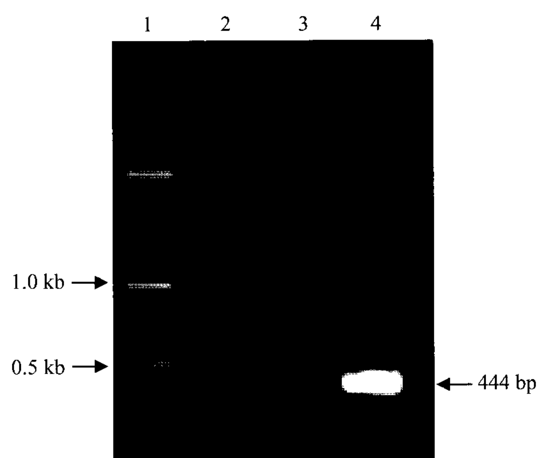

Fig. 2. PCR amplification of the KS domain from *S. toxytricini* chromosomal DNA. Lane 1, 1 kb DNA marker; lane 2, PCR product amplified with F-1/R-1 primers; lane 3, PCR product amplified with F-2/R-1 primers, lane 4; PCR product amplified with F-3/R-1 primers.

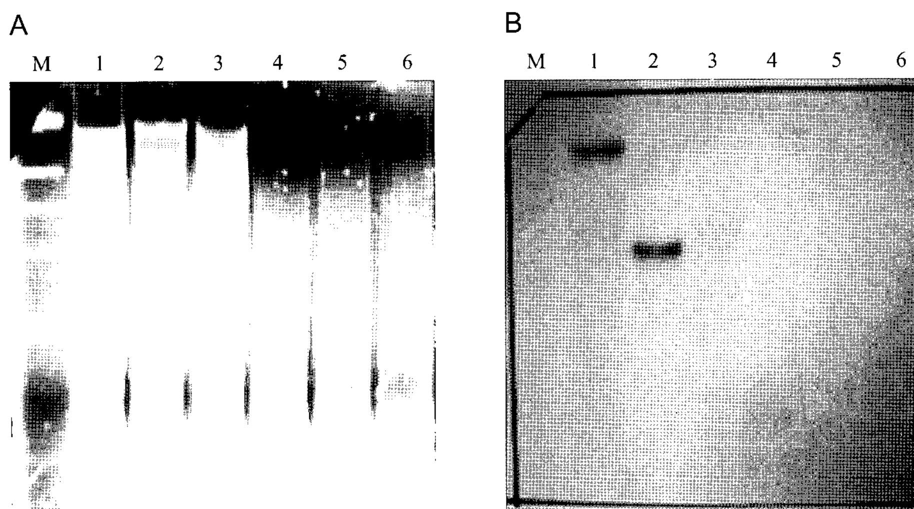

Fig. 3. Southern hybridization of *S. toxytricini* chromosomal DNA using a ^{32}P-labeled KS DNA probe. (A) Agarose gel electrophoretic pattern of *S. toxytricini* chromosomal DNA digested with a restriction enzyme. (B) Southern hybridization of *S. toxytricini* chromosomal DNA after transfer from agarose gel to nylon membrane. Lane 1, λ DNA/*Hin*dIII size marker; lane 2, *S. toxytricini* DNA digested with *Bam*HI; lane 3, *S. toxytricini* DNA digested *Pst*I; lane 4, *S. toxytricini* DNA digested with *Sal*I; lane 5, *S. toxytricini* DNA digested with *Hin*cII; lane 6, *S. toxytricini* DNA digested with *Sac*I; lane 7, *S. toxytricini* DNA digested with *Kpn*I.

chromosomal DNA using primers designed from consensus sequences of KS genes in various type II PKSs, including lovastatin and compactin biosynthetic gene clusters. A 444 bp PCR product was obtained when F-3 and R-1 primers were used (Fig. 2). The nucleotide sequence of the amplified KS gene fragment showed 82% homology with *S. aureofaciens* type II PKS and 75% homology with *S. coelicolor* type II PKS.

Southern blotting of *S. toxytricini* chromosomal DNA with the amplified KS gene fragment revealed a strong hybridization band around 4.5 kb when cut with *Pst*I (Fig. 3). Thus, 4-5 kb fragments of *Pst*I-cut *S. toxytricini* chromosomal DNA were isolated, cloned into a pGEM-3Zf(+) vector, and screened with the amplified KS probe. The isolated clone, the pPKSII plasmid, had a 4.3 kb insert size at the *Pst*I cloning site.

Analysis of type II PKS gene from S. toxitricini

Nucleotide sequencing of the cloned gene revealed four open reading frames (ORFs), which are essential to type II polyketide biosynthesis (Fig. 4, Table 1). The determined sequence has been deposited in the GenBank under the accession number of DQ8554748. The *orf1* gene encodes the 239 amino acids of thioesterase (TE), which showed 56% homology with GrsT of *S. rochei* (Mochizuki *et al.*, 2003) and 53% homology with GrhD of *S.* sp. JP95 (Li and Piel, 2002). A conserved region of the ORF1 protein included the GxSxG motif around the active Ser^{74} residue, which is common to lipases or esterases involved in acyl transfer reactions (Akoh *et al.*, 2004). The *orf2* gene directs the synthesis of β-ketoacyl-ACP synthase (KAS), which consists of 421 amino acids. KAS has a 78% identity with Aur2A of *S. aureofaciens* (Novakova *et al.*, 2004) and 76% identity with MtmP of *S. argillaceus* (Blanco *et al.*, 1996). The ORF2 protein contains the highly conserved sequence of the condensing enzyme active site, GCTSGLD, at Cys^{165}, as well as the highly conserved motif of the active acyltransferase site, GHSLG, at Ser^{343} (Ye *et al.*, 1994; Lombo *et al.*, 1996; Hyun *et al.*, 1997). The *orf3* gene begins with a GTG codon and encodes a 398 amino acid polypeptide. The ORF3 protein was determined to be a chain length factor (CLF), because it is 47% homologous with Aur2B of *S. aureofaciens* (Novakova *et al.*, 2004) and 41% homologous with MtmK of *S. argillaceus* (Blanco *et al.*, 1996). The characteristic lack of the highly conserved Cys residue of the KAS active site in CLF was also identified in this protein (Lombo *et al.*, 1996). However, the diverse region for polyketide chain length control, suggested by Tang *et al.* (2003), was completely absent in this protein. The final open

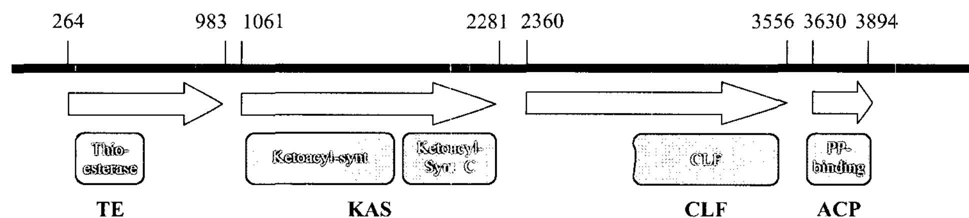

Fig. 4. Organization of the type II PKS gene cluster of *S. toxytricini.* Based on the nucleotide sequence of the type II PKS gene in pPKSII, the four ORFs found by the ORF finder program were thioesterase (TE), β-ketoacyl-ACP synthase (KAS), chain length factor (CLF), and acyl carrier protein (ACP). The arrows indicate the location and direction of each gene. The conserved motifs found using a BLAST search are represented beneath each arrow.

Table 1. Analysis of a type II polyketide synthase gene cluster cloned from *S. toxytricini* chromosomal DNA

Gene	Nucleotide coordinate	Amino acids	Homology (%)	Putative function
orf 1	264-983	239	56	predicted thioesterase GrsT (*S. rochei*)
			53	putative thioesterase GrhD (*S.* sp. JP95)
orf 2	1061-2281	421	78	β-ketoacyl-ACP synthase (*S. aureofaciens*)
			76	ketoacyl synthase MtmP (*S. argillaceus*)
orf 3	2360-3556	398	47	chain length factor-like protein (*S. aureofaciens*)
			41	chain length factor MtmK (*S. argillaceus*)
orf 4	3613-3876	87	50	putative acyl carrier protein GrhC (*S.* sp. JP95)
			50	acyl carrier protein Acp (*S. venezuelae*)

The deduced amino acid sequences of each ORF were compared using the BLAST program in order to determine the putative function of each ORF.

Table 2. Lipstatin production of KAS gene disruptant and parent strain of *S. toxytricini*

	Final pH	Dry cell weight (mg/ml)	Lipstatin production	
			(μg/ml)	(μg/mg)
Parent strain	8.9	3.9	3.62	0.92
Disruptant	8.9	4.5	3.38	0.75

In order to ensure production of lipstatin, the parent strain and gene disruptant of *S. toxytricini* were cultured for 3 days at 28°C in growth media, transferred to production media, and fermented for 3 days at 28°C. The final pH and dry cell weight were measured from the culture broth. Lipstatin was extracted with ethyl acetate and assayed via fluorospectrometry using 4-methylumbelliferyl oleate.

reading frame gene, *orf4*, was characterized as an acyl carrier protein (ACP) gene consisting of 87 amino acids. It exhibits 50% homology both with GrhC of *S.* sp. JP95 (Li and Piel, 2002) and Acp of *S. venezulelae* (Kulowski *et al.*, 1999). The phosphopantetheine attachment site motif around Ser^{41}, LGYxSLxxL, to which a pantetheinyl cofactor is attached, was also found.

Gene disruption of KAS gene

To confirm the involvement of the cloned type II PKS gene cluster in lipstatin biosynthesis, the KAS gene in *orf2* was disrupted using homologous recombination. The obtained exoconjugant was characterized as a double cross-over disruptant due to its neomycin-resistant and apramycin-sensitive phenotype. The fermented broth of the disruptant was extracted with ethyl acetate and the amount of lipstatin produced was determined via a lipase inhibition test using 4-methylumbelliferyl oleate as a substrate (Lim *et al.*, 2006). As shown in Table 2, the KAS disruptant produced nearly the same amount of lipstatin as the *S. toxytricini* wild type. This result implies that the cloned type II PKS gene cluster is not involved in lipstatin synthesis.

Discussion

Lipstatin is produced via Claisen condensation of two fatty acids, 3-hydroxytetradeca-5,8-dienoic acid and hexylmalonic acid, followed by the introduction of *N*-formylleucine at the 5-hydroxyl group (Fig. 1; Eisenreich *et al.*, 1997; Goese *et al.*, 2000; Goese *et al.*, 2001; Schuhr *et al.*, 2002). Based on these findings, we assumed that the two fatty acids forming the lipstatin backbone might be produced by a type II PKS, as in the case of lovastatin or compactin (Hendrickson *et al.*, 1999; Kennedy *et al.*, 1999; Abe *et al.*, 2002). A KS gene fragment of a type II PKS was amplified from *S. toxytricini* chromosomal DNA and used as a probe for the screening of type II PKS gene clusters. Since a 4.5 kb fragment of *Pst*I-digested chromosomal DNA was found to contain a type II PKS gene via Southern blotting, a type II PKS gene was screened from 4-5 kb fragments of the *Pst*I-restricted library using the amplified probe.

The nucleotide sequence determination revealed that the cloned PKS gene cluster comprises three important genes involved in type II polyketide synthesis; KAS, CLF and ACP. However, the cyclase gene responsible for the cyclization of the synthesized polyketides, which is generally found in type II PKS gene clusters, was not found in this clone. In contrast with other type II PKS clusters, a TE gene was observed at upstream of the type II PKS in this clone.

In order to determine whether or not the cloned gene may participate in the production of lipstatin, a KAS gene disruptant was constructed using homologous recombination with a pKC1139 plasmid (Bierman *et al.*, 1992; Yong and Byeon, 2005). The KAS disruptant produced nearly the same amount of lipstatin as the parent strain, which shows that the cloned type II PKS gene cluster is not involved in lipstatin synthesis. Thus it can be concluded that the two fatty acids do not originate from polyketide synthesis, but rather from the degradation of ingested fatty acids.

The culture broth of wild *S. toxytricini* did not show any antibacterial activity against *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis*, *E. coli* XL-1 Blue, or *Pseudomonas aeruginosa* (data not shown), which shows that the cloned type II PKS gene cluster is not involved in antibacterial production. It can also be concluded that the cloned gene cluster does not participate in pigment production, because the wild type colony is colorless. The real biological function of the cloned gene cannot be determined yet because there were no distinguishable morphological differences between wild type and gene disruptant colonies.

Acknowledgement

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