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The role of acyl-coenzyme A carboxylase complex in lipstatin biosynthesis of *Streptomyces toxytricini*

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Abstract *Streptomyces toxytricini* produces lipstatin, a specific inhibitor of pancreatic lipase, which is derived from two fatty acid moieties with eight and 14 carbon atoms. The *pccB* gene locus in 10.6 kb fragment of *S. toxytricini* chromosomal DNA contains three genes for acyl-coenzyme A carboxylase (ACCase) complex *accA3*, *pccB*, and *pccE* that are presumed to be involved in secondary metabolism. The *pccB* gene encoding a β subunit of ACCase [carboxyltransferase (CT)] was identified upstream of *pccE* gene for a small protein of ε subunit. The *accA3* encoding the α subunit of ACCase [biotin carboxylase (BC)] was also identified downstream of *pccB* gene. When the *pccB* and *pccE* genes were inactivated by homologous recombination, the lipstatin production was reduced as much as 80%. In contrast, the accumulation of another compound, tetradeca-5.8-dienoic acid (the major lipstatin precursor), was 4.5-fold increased in disruptant compared with wild-type. It implies that PccB of *S. toxytricini* is involved in the activation of octanoic acid to hexylmalonic acid for lipstatin biosynthesis.

Keywords Acyl-CoA carboxylase · *pccB* · Lipstatin · Octanoic acid · Tetradeca-5.8-dienoic acid · *Streptomyces toxytricini*

Introduction

Acyl-coenzyme A(CoA) carboxylase (ACCase) complex is an important metabolic system, which catalyzes the first committed step in the biosynthesis of fatty acids and polyketides in animals, plants, and bacteria (Cronan and Waldrop 2002). This ubiquitous enzyme is responsible for the activation of various organic acids by α-carboxylation of acyl-CoA that can serve as the building blocks for fatty acid biosynthesis.

In *Streptomyces* and *Mycobacterium* species, the ACCase consists of α, β, and ε subunits (Diacovich et al. 2002; Gago et al. 2006). In α subunit [biotin carboxylase (BC)], there are biotin binding domain and CO_2 fixation domain. A molecule of CO_2 is transferred to the biotin moiety at the C terminus of α subunit to form carboxyl–BC. Subsequently, the β subunit [carboxyltransferase (CT)] transfers the carboxyl group from carboxyl–BC to the acyl-CoA (Cronan and Waldrop 2002). Because the acyl-CoA participates only in the second step, the CT determines the substrate specificity of ACCase complex recognizing different acyl-CoAs.

Two types of ACCases, acetyl-CoA carboxylase (ACC) and propionyl-CoA carboxylase (PCC) have been characterized in *Streptomyces coelicolor* (Diacovich et al. 2002; Rodriguez and Gramajo; 1999; Rodriguez et al. 2001). It has been revealed that those ACCases have different roles in metabolism; ACC plays role in primary metabolism for fatty acid synthesis and PCC in secondary metabolism for polyketide synthesis. A recent study showed that the β

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subunit encoded by *pccB* gene and ε subunit by *pccE* gene in *S. coelicolor* form a protein complex, which exhibited full activity with longer chain acyl-CoA rather than acetyl-CoA (Diacovich et al. 2002).

Lipstatin isolated from *Streptomyces toxytricini* is a potent irreversible inhibitor of pancreatic lipase (Weibel et al. 1987; Hochuli et al. 1987). The data provided from the feeding experiments and tracer studies indicated that the carbon skeleton of lipstatin molecule is biosynthesized via Claisen condensation of two fatty acid precursors, 8-carbon atoms (octanoic acid), and 14-carbon atoms (tetradeca-5,8-dienoic acid; Fig. 1; Eisenreich et al. 1997; Goese et al. 2000, 2001;Schuhr et al. 2002; Eisenreich et al. 2003).

The reaction producing an ultimate 3-oxo or hydroxy intermediate before the β–lactone formation in lipstatin (Goese et al. 2001) resembles the biosynthesis of a mycolic acid (Portevin et al. 2005). A gene knock-out study in *Corynebacterium diphtheriae* demonstrated that the *pks13* gene flanked by two other genes, *fadD32* for acyl-AMP ligase and *accD4* for ACCase, is responsible for the condensation step to form mycolic acid (Portevin et al. 2004). This Pks13 condensase is a non-iterative type I polyketide synthase that contains four catalytic domains

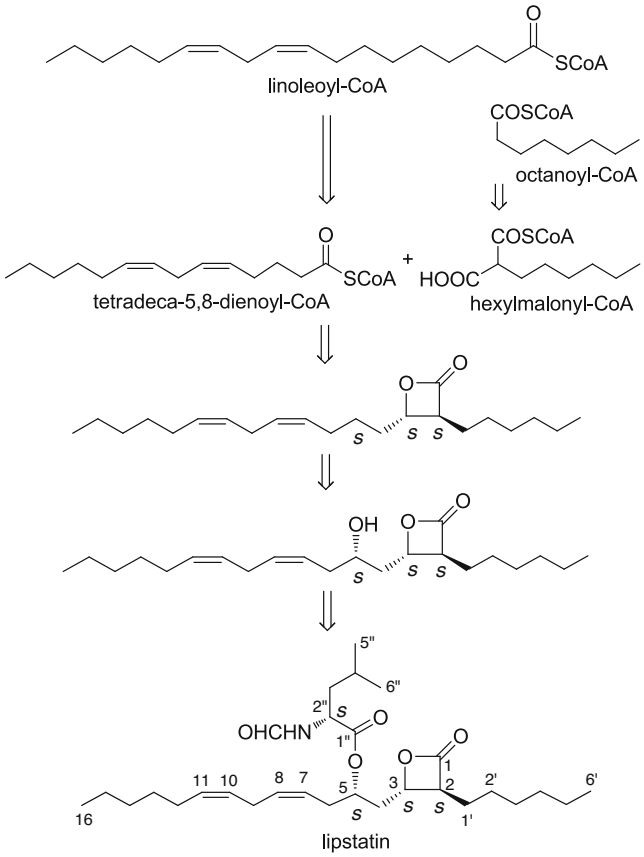

Fig. 1 Hypothetical biosynthetic pathway of lipstatin. The lipstatin backbone is formed via Claisen condensation of two fatty acid precursors, octanoic acid, and tetradeca-5,8-dienoic acid

required for the final assembly of mycolic acid from two fatty acid residues. Later the *accD4* gene was also confirmed to activate one of the mycolic precursor (Portevin et al. 2005).

Based on the structural similarity between the mycolic acids and 3-hydroxo intermediate of lipstatin, the *pks13* gene was screened from *S. toxytricini* chromosomal DNA but any positive result was not obtained. Instead, *pccB* gene flanked by *accA3*, *pccE*, and *bpl* genes was identified based on an *accD4* gene sequence. The disruption experiment showed that this gene locus is involved in the activation of octanoic acid before generation of lipstatin skeleton by Claisen condensation. Here, we report the biochemical role of *pccB* gene locus encoding PCC complex in lipstatin biosynthesis.

Materials and methods

Bacterial strains, culture media, and cultivation

The *Escherichia coli* strains used in this study were listed in Table 1. The *E. coli* strains were generally grown overnight at 37 °C in Luria-Bertani (LB) medium supplemented with appropriate antibiotics (Sambrook and Russell 2001), but *E .coli* XL1-Blue MRF′ was cultured in LB broth supplemented with 10 mM $MgSO_4$ and *E. coli* BW25113/pIJ790 and ET12567/pUZ8002 in SOB–$MgSO_4$ media (Datsenko and Wanner 2000).

Wild-type of *S. toxytricini* NRRL 15443 was grown on modified mannitol soya flour (MS) or ISP2 agar plates or in tryptic soy broth (TSB) liquid medium at 29 °C. For cultivation of the exoconjugant containing disruption cassette, a normal or modified MS agar plates were supplied with either apramycin/kanamycin or apramycin alone (Datsenko and Wanner 2000). A spore suspension of *S. toxytricini* strains was inoculated in seed culture medium (per l; soya bean flour 10 g, glycerol 5 ml, Bacto soytone 5 g, soya oil, 10 ml, Triton X-100 2 ml, pH 6.5) and agitated at 29 °C for 48 h under aerobic conditions. This seed culture (3%) was transferred into fermentation medium (per l; soya bean flour 30 g, glycerol 14 ml, Bacto soytone 1 g, Tryton X-100 1 ml, polypropylene glycol 0.2 ml, soya oil 60 ml, pH 7.0) for lipstatin production. Fermentation was carried out at 29 °C for 6.5 days under aerobic condition.

DNA manipulation

Isolation of chromosomal and plasmid DNA, agarose gel electrophoresis, restriction digestion and ligation, preparation of competent cell, and plasmid transformation were performed by conventional methods (Kieser et al. 2000; Sambrook and Russell 2001).

Table 1 Bacterial strains and plasmids used in this study

Strains	Genotype/related characteristics	Reference
E. coli strains		
E. coli DH5α	*supE*44 Δ*lac*U169 (*Φ*80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	(Hanahan 1983)
E. coli ET12567	*F*⁻ *dam*-13::Tn*9* *dcm*-6 *hsdM hsdR*	(MacNeil et al. 1992)
E. coli XL1-Blue MRF′	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi*-1 *relA*1 *lac*⁻ *Δ(mcrA)*^{183} Δ(*mcrCB-hsdSMR-mrr*) 173F′ [*proAB*$^{+}$ *lacI*^{q} *lacZ* ΔM15 *tet*^{r}]	Stratagene, USA
E. coli BW25113	*lacI*^{8} Δ*lacZ4787 hsdR514* Δ(*araBAD*)*567* Δ(*rhaBAD*)*568 rph-1 rpo5369*	(Datsenko and Wanner, 2000)
S. toxytricini strains		
S. toxytricini NRRL15443	Wild strain, the lipstatin producer	USDA
S. toxytricini BE-AP	Mutant strain disrupting *pccB* and *pccE* genes with *aac(3)IV*	This study
Plasmids		
pGEM-3Zf(+)	ColE1 *ori*f1 *ori*, *bla*, *lacZ-α*	Promega, USA
pGEM-5Zf(+)	ColE1 *ori*f1 *ori*, *bla*, *lacZ-α*	Promega, USA
pG3-P4.8	4.8 kb *Pst*I insert in pGEM-3Zf(+), containing part of *pccB*, *pccE*, *maf*, and *accA*3 upstream	This study
pG5-N3.5	3.5 kb *Nco*I insert in pGEM-5Zf(+), containing part of *bpl* , *pccB*, and *pccE*	This study
pOJ446	SCP2* *ori*, pBR *ori*, *aac(3)IV*, *λ cos, oriT* (RK2), *Streptomyces – E. coli* shuttle cosmid vector	(Bierman et al. 1992)
pUZ8002	*aph*, RK2 derivative with defective *oriT*; non-transmittable plasmid	(Paget et al. 1999)
pIJ790	*araC bet cat exo gam repA101*^{ts} P*araBAD*, *ori*R101, derivative of the λ-Red recombination plasmid pKD20	(Datsenko and Wanner, 2000)
pIJ773	*bla, aac(3)IV*, *lacZα, oriT* (RK2) flanked by FRT sites; pBluescript KS(+) derivative	(Datsenko and Wanner, 2000)
pHZ1351	*ori* (pIJ101), *oriT* (RK2), *cos*, *bla*, *ori* (ColE1), *sti* (pIJ101), *tsr Ltz*⁻, *rep* (pIJ101) recombination–prone unstable plasmid used in the gene disruption	(Sun et al. 2002)
pHZ-BE24	pHZ1351 having a 2.78 kb insert of *pccB* and *pccE* genes	This study
pHZ-BE-Ap	Gene replacement vector for *pccB-pccE* genes, containing disruption cassette of *acc(3)IV* and *oriT* in pHZ1351	This study

PCR amplification

The gene amplification was done using EF-*Taq* DNA polymerase or *Pfu* DNA polymerase (Solgent, Korea) according to the supplier's protocol. For the preparation of *pccB* probe, the PCR was performed at 58 °C annealing temperature with the combinations of two sets of forward and reverse primers accB–F1 (5′-CCGRTYRT CGGCATY AACGACTC-3′), accB–F2 (5′-AVGAYTCYGGYG GYGCHCGYATCCA-3′), accB–R1 (5′-TGCTTGGAB CCCATSACSKCGTA-3′), and accB–R2 (5′-GABCCCAT SACSKCRTAVG CDCCGCC-3′).

Construction of *S. toxytricini* genomic library

The chromosomal DNA was isolated from mycelia of *S. toxytricini* using a cetyltrimethylammonium bromide procedure (Kieser et al. 2000) and partially digested with *Sau*3AI. The large-size DNA fragments (30–45 kb) were eluted and ligated with the cosmid vector pOJ446 (Bierman et al. 1992) which was digested with *Hpa*I, dephosphorylated, and further cleaved by *Bam*HI. The ligated DNA products were packaged *in vitro* by Gigapack III gold packaging extract (Stratagene, USA), and transfected into *E. coli* XL1-Blue MRF′ cells, to construct the cosmid library of *S. toxytricini* chromosomal DNA.

Southern hybridization

The probe was labeled with α-^{32}P-dCTP (specific activity; 50 μCi, Amersham, UK) using DecaLabel DNA labeling kit (Fermentas, Lithuania) according to manufacturer's specification and purified through Elutip-D column (Schleicher and Shuell, Germany). The capillary Southern hybridization or *in situ* colony hybridization was performed by conventional procedure (Sambrook and Russell 2001).

DNA sequencing and analysis

The selected recombinant plasmids were sent to Solgent (Daejeon, Korea) for sequencing. The location of open

reading frame (ORF)s in the sequences was determined using FramePlot version 2.3.2. The ORFs were translated into amino acid sequences using translate tool program (http://br.expasy.org/tools/dna.html). The homology search with the obtained DNA or protein sequences was performed with BLAST program (http://blast.ncbi.nlm.nih.gov). The multiple alignments of DNA or protein sequences were performed with EBI–ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Gene disruption by PCR targeting system

A DNA fragment containing the two genes *pccB* and *pccE* was amplified from cosmid pSTL24 by *Pfu* DNA polymerase using primers: BE-up F1 (5′-GCGGATCCGA GGTCGGTGTTGGTGGAGCCCGT–3′) having *Bam*HI site and BE-dn R2 (5′-GCAAGCTTGTCGCCGTGTC GACCACGCAGTG–3′) having *Hind*III site. The resulting 2.78 kb PCR product was digested with *Bam*HI and *Hin*dIII and ligated at the same restriction sites of pHZ1351 plasmid (Sun et al. 2002) to give pHZ-BE24 plasmid (10.8 kb). The confirmed plasmid was transformed again by electroporation into *E. coli* BW25113/pIJ790 for λ-Red recombination and PCR-targeted gene disruption (Gust et al. 2003). The apramycin resistance cassette having *aac(3)IV* in plasmid pIJ773 was amplified with two long primers, PCCB Red F1 (5′-GAGTTCGCCC GGCACCGGTCGACCAACTTCGGCTGGAG*ATTCC GGGGATCCGTCGACC*–3′) and PCCB Red R1 (5′–GGGTACTCAGGCGCCGCCTCCGCTTCGCG CCGTCTCTCA*TGTAGGCTGGAGCTGCTTC*–3′) having 19 or 20 nucleotides of target genes at 3′-end flanked FLP recognition targets (FRT). The extended apramycin disruption cassette was introduced by electroporation into *E. coli* BW25113/(pIJ790 and pHZ-BE24) grown in SOB–$MgSO_4$ containing chloramphenicol and ampicillin with supplementation of 10 mM L-arabinose for the gene replacement by inducing of λ-Red recombinase genes. Immediately after electroporation, 1 ml of ice-cold LB was added to the shocked cells and incubated with shaking for 1.5 h at 30 °C. The revived cells were spread on LB agar plates containing ampicillin and apramycin, followed by incubation at 37 °C to promote the loss of temperature-sensitive plasmid pIJ790 having $repA101^{ts}$. The newly obtained pHZ-BE-Ap plasmid contained the apramycin disruption cassette instead of the target genes. This apramycin disruption vector was then transformed by electroporation into *E. coli* ET12567/pUZ8002 to construct a donor strain for its conjugal transfer to *S. toxytricini* wild strain. The *S. toxytricini* spores preserved in sterile 20% glycerol and 0.01% Triton X-100 were resuspended in 500 μl of TES buffer (0.05 M *N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid, pH 8.0), heat-shocked at 50 °C for 10 min for germination, and then mixed with equal volume of *E. coli* ET12567/(pUZ8002 and pHZ-BE-Ap) cells (Flett et al. 1997). After plating on MS agar and incubation at 30 °C for 16–40 h, 1 ml solution containing 0.5 mg of nalidixic acid and 1.25 mg apramycin was overlaid on the plate and the incubation continued at 29 °C for 3–5 days until potential exoconjugants sensitive to kanamycin but resistant to apramycin grew up.

Metabolite separation and analysis

The lipstatin metabolites were isolated from fermentation broth using standard purification method (Erdei et al. 2005). First 100 ml fermentation broth was extracted with 500 ml of acetone−hexane (1.5:1), dried over sodium sulfate, and concentrated to oil form. The crude oil was further separated by Silica 60 gel (Merck, Germany) column chromatography by eluting with hexane and then with hexane−ethyl acetate mixture in the ordered ratio of 20:1, 10:1, 5:1, and 2.5:1. The fractions were monitored by thin liquid chromatography (TLC) on Silica 60 F_{254} aluminum sheets (Merck, Germany), which were developed in hexane–ethyl acetate (2.5:1) and visualized with 10% sulfuric acid. The fractions showing spots of the same Rf values were combined and concentrated to dryness. Further metabolites were separated by high performance liquid chromatography (HPLC) (Souri et al. 2007) using SCL-$10A_{VP}$ system (Shimadzu Co., Japan) with YMC-Pack ODS-A reverse-phase column (250 mm× 10 mm internal diameter (l.D.), 12 nm; YMC Co., Ltd., Japan). The isocratic mobile phase of 0.1% orthophosphoric acid–acetonitrile (15:85 *v*/*v*) was pumped at a flow rate of 1 ml/min, and the metabolites were detected at 205 nm. The separated pure metabolites by preparative HPLC were subjected to ^{1}H-nuclear magnetic resonance (NMR) for the determination of chemical structures. The samples were dissolved in chloroform-d_1, and the NMR spectra were recorded on Bruker AMX spectrometer (Bruker Biospin, Germany) using Bruker's standard pulse program for ^{1}H NMR (250 MHz, FT).

Determination of lipase inhibitory activity

The inhibitory activity of *S. toxytricini* metabolites on human pancreatic lipase was assayed with 4-methylumbelliferyl oleate as substrate following the procedure described previously (Imanaka et al. 1983). The fluorescence intensity of 4-methylumbelliferone liberated from the substrate was measured with a fluorescence microplate reader (FLUOstar OPTIMA, BGM Labtechnologies, Germany) using excitation at 340 nm and emission at 460 nm.

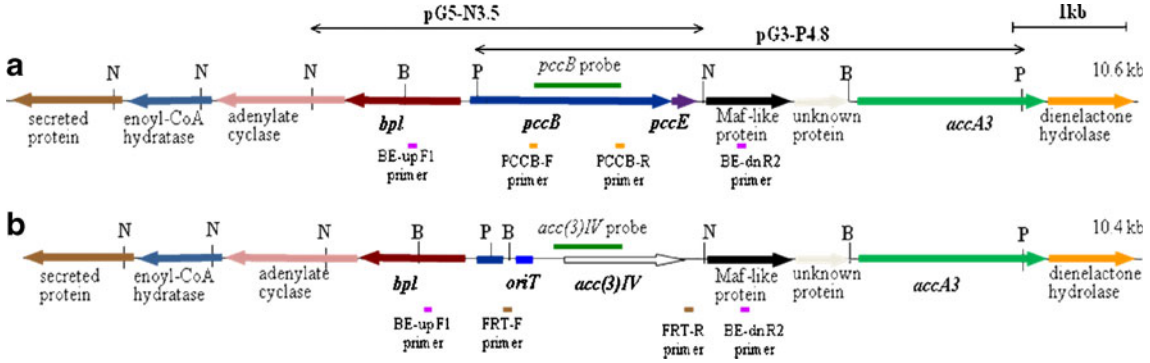

Fig. 2 Gene organization of *pccB* locus (10.6 kb) in the wild-type *S. toxytricini* (**a**) and in the disruptant *S. toxytricini* BE-AP strain having apramycin resistance gene [*acc(3)IV*] in place of *pccB* and *pccE* genes (**b**). The probes used in Southern hybridization are presented above the respective location, and the primers are denoted below the locations. The restriction sites are shown as abbreviations—N for *Nco*I; B for *Bam*HI, and P for *Pst*I

Results

Cloning of *pccB* gene locus from *S. toxytricini* chromosomal DNA

The two conserved regions were found in the sequence alignment of AccD4 of *Mycobacterium* species and PccB of *Streptomyces* species and used to design primers capable of amplifying a DNA fragment with size of 880 bp. The amino acid sequence of the amplified gene fragment showed high level of similarity with PccB of *Streptomyces avermetilis* MA-4680 (91%) and *S. coelicolor* A3(2) (89%), as well as AccD4 of *Mycobacterium tuberculosis* H37Rv (52%), *Mycobacterium bovis* (52%), and *Mycobacterium leprae* (51%).

A cosmid library of *S. toxytricini* genomic DNA constructed in pOJ446 vector was transfected in *E. coli* XL1-Blue MRF′ and screened by *in situ* hybridization with ^{32}P-labeled *pccB* gene fragment as a probe. Southern blot analysis of three positive cosmid clones after digestion with *Pst*I or *Nco*I revealed the presence of 4.8 kb or 3.5 kb DNA bands containing *pccB* gene, respectively (Fig. S1). These

Table 2 Gene organization in the sequenced *pccB* gene locus

ORF	Nucleotide position	Amino acids	Gene designation	Deduced putative function from the BLAST search	Homology (%)
orf1	116-1231	371		Secreted protein [*Streptomyces* sp. Mg1]	84
				Secreted protein [*Streptomyces hygroscopicus* ATCC 53653]	76
orf2	1335-2045	236		Enoyl-CoA hydratase [*Streptomyces* sp. Mg1]	91
				Enoyl-CoA hydratase [*Streptomyces ghanaensis* ATCC 14672]	84
orf3	2154-3332	392		Adenylate cyclase [*Streptomyces* sp. Mg1]	96
				Adenylate cyclase [*Streptomyces clavuligerus* ATCC 27064]	86
orf4	3460-4338	292	*bpl*	Biotin apo-protein ligase [*Streptomyces* sp. Mg1]	83
				Biotin apo-protein ligase [*Streptomyces avermitilis* MA-4680]	71
orf5	4470–6068	533	*pccB*	Putative acyl-CoA carboxylase β-subunit [*Streptomyces* sp. Mg1]	94
				Propionyl-CoA carboxylase [*Streptomyces pristinaespiralis* ATCC 25486]	91
orf6	6097–6312	71	*pccE*	Hypothetical protein [*Streptomyces coelicolor* A3(2)]	66
				Hypothetical protein [*Streptomyces avermitilis MA-4680*]	69
orf7	6556–7167	203		Putative serum formation protein [*Streptomyces avermitilis MA-4680*]	80
				Putative hypothetical protein [*Streptomyces coelicolor* A3(2)]	72
orf8	7313-7756	147		Unknown (no homologous protein)	
orf9	7988–9751	587	*accA3*	Acyl-CoA carboxylase complex A subunit [*Streptomyces* sp. Mg1]	93
				JadJ [*Streptomyces pristinaespiralis* ATCC 25486]	91
orf10	9867-10592	241		Dienelactone hydrolase [*Streptosporangium roseum* DSM 43021]	87
				Conserved hypothetical protein [*Streptomyces* sp. Mg1]	85

two bands hybridized with *pccB* probe were cloned at *Pst*I site of pGEM-3Zf(+) to construct pG3-P4.8 plasmid or *Nco*I site of pGEM-5Zf(+) to give a pG5-N3.5 plasmid.

The nucleotide sequences of these two DNA fragments were assembled manually in a single contig DNA sequence of 6.2 kb and further extended its upstream and downstream to 10.6 kb. The resulted nucleotide sequence was deposited in GenBank under the accession number of FJ595232.

Organization of *S. toxytricini pccB* gene locus

This DNA sequence named as the *pccB* gene locus comprises ten distinct ORFs including *bpl*, *pccB*, *pccE,* and *accA3* genes for ACCase complex (Fig. 2a, Table 1). Four genes including *bpl* is located upstream of *pccB* gene and expressed in opposite direction. The other six genes including *pccB*, *pccE*, and *accA3* are with the same orientation (Table 2).

Multiple alignment of *S. toxytricini* PccB with other *Streptomyces* PccB and *Mycobacterium* AccD5 revealed the presence of acyl-CoA binding site and biotin binding site (Fig. 3a). The structural analysis of PccB elucidated the involvement of the $GG^{182\text{-}183}$ residues in hydrogen bonding with the carbonyl group of acyl-CoA, whereas $GA^{419\text{-}420}$ residues in hydrogen bonding with carbonyl group of biotin (Diacovich et al. 2004; Lin et al. 2006). The substrate specificity of PCC has known to be dependent upon the residue located outside of substrate pocket; D^{422} of *Streptomyces* PccB or C^{435} of *Mycobacterium* AccD5. The *S. toxytricini* PccB has the same amino acids at substrate binding pocket as other *Streptomyces* PccB proteins.

In the translated AccA3, the ATP-binding motif and CO_2 fixation site as well as the conserved MKM motif for biotinylation were present (Fig. 3b). The sequence $GGGGRGLKVAR^{161\text{-}171}$ matched the consensus sequence of Gly-rich motif for ATP-binding in all α subunits (BC;

Fig. 3 Amino acid sequence alignment of homologous regions in the active site of the putative PccB (**a**), AccA3 (**b**), PccE (**c**), and Bpl (**d**) proteins encoded by genes in *pccB* locus of *S. toxytricini*. StxPccB, PccB of *S. toxytricini* NRRL 15443; ScoPccB, PccB of *S. coelicolor* A3(2) (NP_629079), SavPccB; PccB of S. *avermitilis* MA-4680 (NP_824507); SprPccB, PccB of *S. pristinaespiralis* ATCC 25486 (ZP_03173623); SclPccB; PccB of *S. clavuligerus* ATCC 27064 (ZP_03182649), MtbAccD5, AccD5 of *Mycobacterium tuberculosis* H37Rv (NP_217797); MboAccD5, AccD5 of *M. bovis* AF 2122/97 (NP_856953); MleAccD5, AccD5 of *M. leprae* TN (NP_301571); CdpAccD5, AccD5 of *C. diphtheriae* NCTC 13129 (NP_939032), StxAccA3, AccA3 of *S. toxytricini* NRRL 15443; ScoAccA3, *S. coelicolor* A3(2) (NP_629074); SavAccA3, AccA3 of *S. avermitilis* MA-4680 (NP_824513); SprJadJ, JadJ of *S. pristinaespiralis* ATCC 25486 (ZP_05009963); SclJadJ; JadJ of *S. clavuligerus* ATCC 27064 (ZP_05005117); MtbAccA3; AccA3 of *M. tuberculosis* H37Rv (NP_217802); MboAccA3; AccA3 of *M. bovis* AF 2122/97 (NP_856958); MleBccA; BccA of *M. leprae* TN (NP_301567); CdpAccBC; of *C. diphtheriae* NCTC 13129 (NP_939023); StxPccE; PccE of *S. toxytricini* NRRL 15443, ScoPccE; PccE of *S. coelicolor* A3(2) (NP_629078); SavPccE; PccE of *S. avermitilis* MA-4680 (NP_824508); SprPccE; PccE of *S. pristinaespiralis* ATCC 25486 (YP_002201271); SclPccE, *S. clavuligerus* ATCC 27064 (ZP_03182650); StxBpl, Bpl of *S. toxytricini* NRRL 15443, SavBirA; BirA of *S. avermitilis* MA-4680 (NP_824506); ScoBpl; Bpl of *S. coelicolor* A3(2) (NP_629080); SclBpl; Bpl of *S. clavuligerus* ATCC 27064 (YP_002193370); SprBpl, Bpl of *S. pristinaespiralis* ATCC 25486 (YP_002201269), MtbBirA, BirA of *M. tuberculosis* H37Rv (NP_217796); MboBirA, BirA of *M. bovis* AF 2122/97 (NP_856952); MleBirA; BirA of *M. leprae* TN (NP_301572); CdpBirA, BirA of *C. diphtheriae* NCTC 13129 (NP_939035)

Kimura et al. 2000). The Cys residue of $RDCS^{226-229}$ involved in CO_2 fixation and $MKM^{551-553}$ motif for biotinylation were also found.

It was already demonstrated that the PccB protein in *S. coelicolor* exhibited higher activity in activation of propionyl-CoA in the presence of PccE subunit (Diacovich et al. 2002). The gene organization in *pccB* gene locus suggests that those genes might be in a functional relationship and be involved in the activation of acyl-CoAs with longer chain than acetyl-CoA, which can be employed as substrates for biosynthesis of secondary metabolites in *S. toxytricini*.

The *bpl* gene encodes a biotin protein ligase transferring biotin molecule to the Lys residue in MKM motif of BC (α subunit; AccA3), which is called a post-translational biotinylation of apo-BC to holo-BC. Residues STN^{54-56}, S^{99}, K^{154}, $GIL^{157-159}$, and N^{174} forming strong hydrogen bonds with biotin molecule (Bagautdinov et al. 2008) were found in the sequence (Fig. 3d). The sequence $GRGR^{80-83}$ for ATP-binding is well-conserved.

Two potential ORFs were identified between *pccB* and *accA3* genes. One resembles *Bacillus maf* gene involved in arrested septum formation (Butler et al. 1993) with residues S^{14}, S^{16}, R^{19}, E^{39}, K^{58}, D^{77}, and K^{89} lying on phosphate ion binding pocket of Maf protein (Minasov et al. 2000), but its real biological function is still uncertain. The other gene encodes polypeptide of 147 amino acids, but there was no similar protein found in the BLAST search.

Gene disruption of the *pccB* locus in *S. toxytricini*

To confirm the involvement of cloned *pccB* gene cluster in lipstatin biosynthesis, insertional inactivation of the *pccB* and *pccE* genes by homologous recombination was attempted (Gust et al. 2003). The amplified DNA fragment of target genes (*pccB* and *pccE*) was inserted in pHZ1351 to construct pHZ-BE24 vector and replaced by homologous recombination with the extended apramycin disruption cassette [*acc(3)IV*] flanked by FRT sites which was amplified from pIJ773. After the newly obtained disruption plasmid named pHZ-BE-Ap was introduced into *E. coli* ET12567/pUZ8002, it was transferred conjugally to wild-type *S. toxytricini* to replace the *pccB* and *pccE* genes in chromosomal DNA by homologous recombination (Flett et al. 1997).

Several brownish *S. toxytricini* exoconjugants were observed on MS agar supplemented with nalidixic acid and apramycin. The double-crossover recombinants were confirmed by a replica plate of the selected clones on nutrient agar supplemented with kanamycin. A single positive clone, resistant to apramycin but sensitive to kanamycin, was selected and designated as *S. toxytricini* BE-AP.

The double-crossover in disruptant *S. toxytricini* BE-AP strain was confirmed by Southern hybridization. Wild strain chromosomal DNA digested by *Bam*HI gave strong signal at 4.0 kb DNA fragment after hybridization with *pccB* probe, while the disruptant did not give any signal (Fig. 2b,

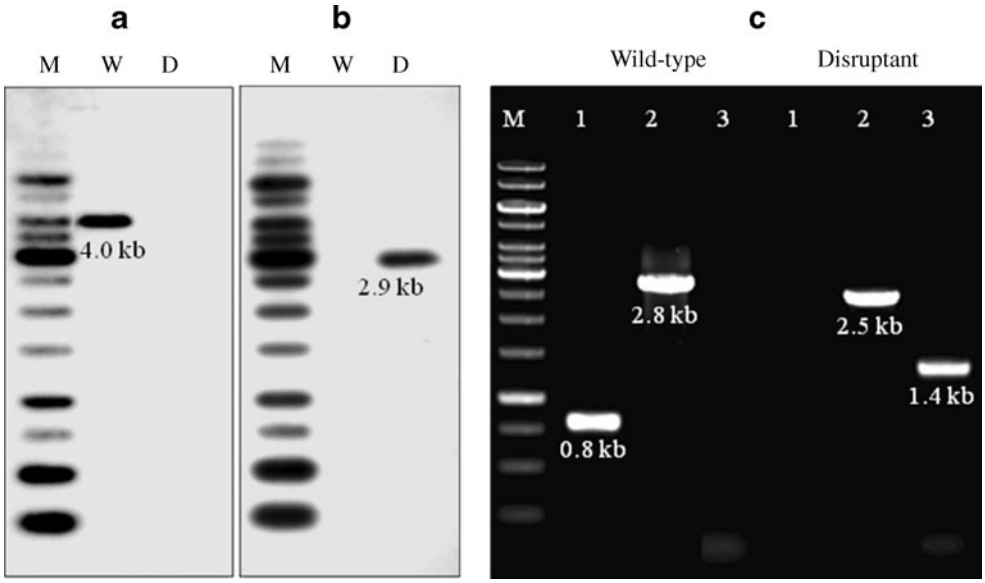

Fig. 4 Confirmation of the double-crossover in *S. toxytricini* BE-AP strain by Southern hybridization and PCR amplification. **a** Southern hybridization pattern of *Bam*HI-digested chromosomal DNA with *pccB* probe. *Lane M*, 1 kb DNA ladder; *lane W*, wild-type strain; *lane D*, BE-AP disruptant. **b** Southern hybridization pattern with *aac(3)IV* probe. *Lane M*, 1 kb DNA ladder; *lane W*, wild-type strain; *lane D*, BE-AP disruptant. **c** PCR amplification of chromosomal DNA from wild-type and BE-AP disruptant. *Lane 1*, amplification using PCCB-F and PCCB R primers producing a 0.8 kb DNA fragment only in the wild-type strain; *lane 2*, gene amplification using BE-up F1 and BE-dn R2 primers giving 2.8 kb DNA band in wild-type and 2.5 kb in the disruptant; *lane 3*, amplification using FRT-F and FRT-R primers giving a 1.4 kb PCR fragment only in disruptant; *lane M*, 1 kb DNA ladder

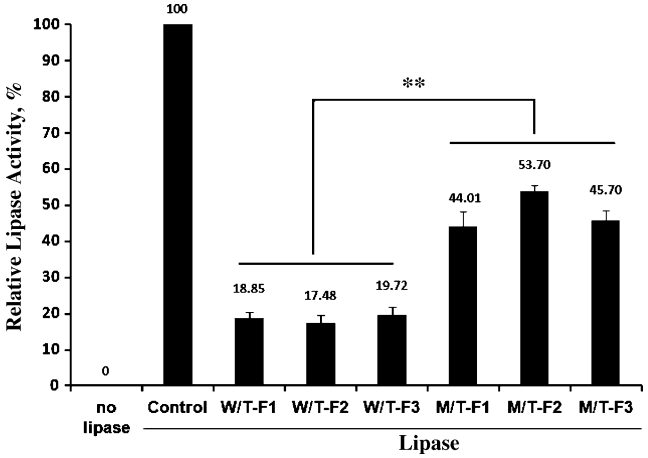

Fig. 5 Lipase inhibitory activity of the crude extracts from *S. toxytricini* wild-type and BE-AP mutant. W/T-F, three independent extracts of wild-type strain; M/T-F, three independent extracts of BE-AP disruptant strain. The *bar graphs* represent the mean ± SD of three independent experiments. Lipase inhibitory activity of 50,000-fold diluted extracts was determined by measuring the fluorescence intensity of 4-methylumbelliferon liberated in the lipase reaction with 4-methylumbelliferyl oleate. $^{**}P<0.001$ compared between the mean values of wild-type and disruptant groups

Fig. 4a, b). Meanwhile, in another hybridization using *acc (3)IV* probe, the chromosomal DNA of BE-AP strain gave a strong signal of 2.9 kb DNA, while the wild strain did not.

PCR amplification of the chromosomal DNAs of the wild-type and BE-AP strains using three different primer set combinations also confirmed the double-crossover (Fig. 2b, Fig. 4c). The combination primer of BE-up-F1 and BE-dn-R2 produced a 2.8 kb DNA band from wild-type chromosomal DNA, but gave a smaller 2.5 kb band from disruptant chromosomal DNA. The 0.8 kb DNA fragment amplified by PCCB-F and PCCB R primers confirmed the presence of *pccB* gene only in the wild-type, whereas the 1.4 kb PCR fragment obtained with FRT-F and FRT-R primers evidenced the replacement by double-crossover with *acc(3)IV* gene only in the disruptant chromosomal DNA.

The role of *pccB* gene locus in lipstatin biosynthesis

The wild-type and BE-AP disruptant were fermented for lipstatin production at 29 °C for 6.5 days under aerobic conditions. The final culture pH of disruptant (pH 6.22± 0.09) was lower than that of the wild-type (pH 6.50±0.03).

The fermentation broths were extracted with acetone–hexane. After extraction, the crude oil obtained from 100 ml of fermentation broth was 4.80±0.05 g in case of wild-type and 5.30 g±0.21 in case of disruptant. The crude extracts were subjected to the inhibition assay for pancreatic lipase. The 50,000-fold diluted crude extracts from the disruptant exhibited lower inhibitory activity than the wild-type crude extracts (Fig. 5). However, the disruptant extracts still inhibited the pancreatic lipase, which implies that the production of the lipase inhibitor was reduced but not completely hindered in the disruptant strain.

Both crude extracts of wild-type and BE-AP strain were separated on Silica 60 gel column chromatography, and the metabolites were identified by TLC and further isolated by HPLC (Fig. 6). In HPLC, two major peaks appeared at

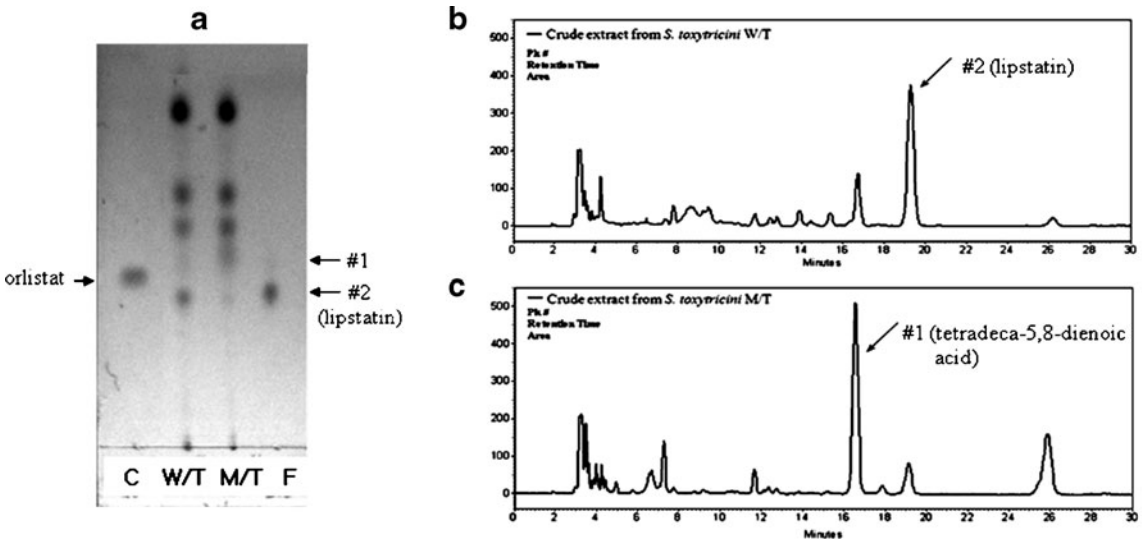

Fig. 6 TLC and HPLC analysis of the crude extracts from *S. toxytricini* wild-type and disruptant strains. **a** TLC pattern of crude extracts on Silica 60 F_{254} sheet. C, orlistat (tetrahydrolipstatin–2 μg/ml); *W/T*, crude extract of wild-type strain; *M/T*, crude oil extract from BE-AP disruptant strain; *F*, fraction of wild-type extract showing the highest lipase inhibition activity which was eluted from Silica 60 column. **b** HPLC pattern of wild-type extract. **c** HPLC pattern of disruptant extract. HPLC analysis was performed by isocratic phase of 0.1% orthophosphoric acid and acetonitrile (15:85) and flow rate of 1 ml/min on C_{18} reverse-phase column. The HPLC chromatograms show two major peaks for compound 1 at 16.6 min and compound 2 at 19.3 min

retention times of 16.6 min (compound 1) and 19.3 min (compound 2). Compound 1 was a major one in the disruptant extract, while compound 2 in the wild-type extract. The peak area calculation showed that the compound 1 increased by four times, whereas, the compound 2 was reduced with 80% in the disruptant extract compared with the wild-type extract.

The two compounds were purified by preparative HPLC from wild-type extracts, and their chemical structures were determined by ^{1}H-NMR (Fig. 7). The ^{1}H-NMR spectrum of the purified compound 2 showed the same spectrum as lipstatin reported by Hochuli et al. (1987). The protons for the unsaturated double bonds were shifted at 5.5 ppm as multiplet and the protons in the lactone ring at 3.2 and 4.3 ppm as multiplets. The characteristic chemical shifts of the protons in the formylleucine residue also appeared at 8.2 ppm for 2″-amino group and at 5.9 ppm for the aldehyde group. The ^{1}H-NMR of the compound 1 revealed to be tetradeca-5.8-dienoic acid, a precursor of lipstatin, which was confirmed by the earlier reported spectrum (Goese et al. 2000).

Discussion

The formation of lipstatin via Claisen condensation (Goese et al. 2001; Eisenreich et al. 2003; Schuhr et al. 2002) resembles the generation of mycolic acid via condensation of two fatty acids by Pks13 (Portevin et al. 2005). However, any attempts to identify a *pks13* (type I *pks*-like gene) in *S. toxytricini* did not bring the expected results (data not shown). Only a type II *pks* gene was previously identified in this strain (Yoo et al. 2006).

Alternatively, the *accD* gene which is assumed to recognize and activate octanoic acid, one of the main precursors for lipstatin, was selected as the target one. Two consecutive attempts to identify such *accD* genes in *S. toxytricini* chromosome lead to identification of two gene

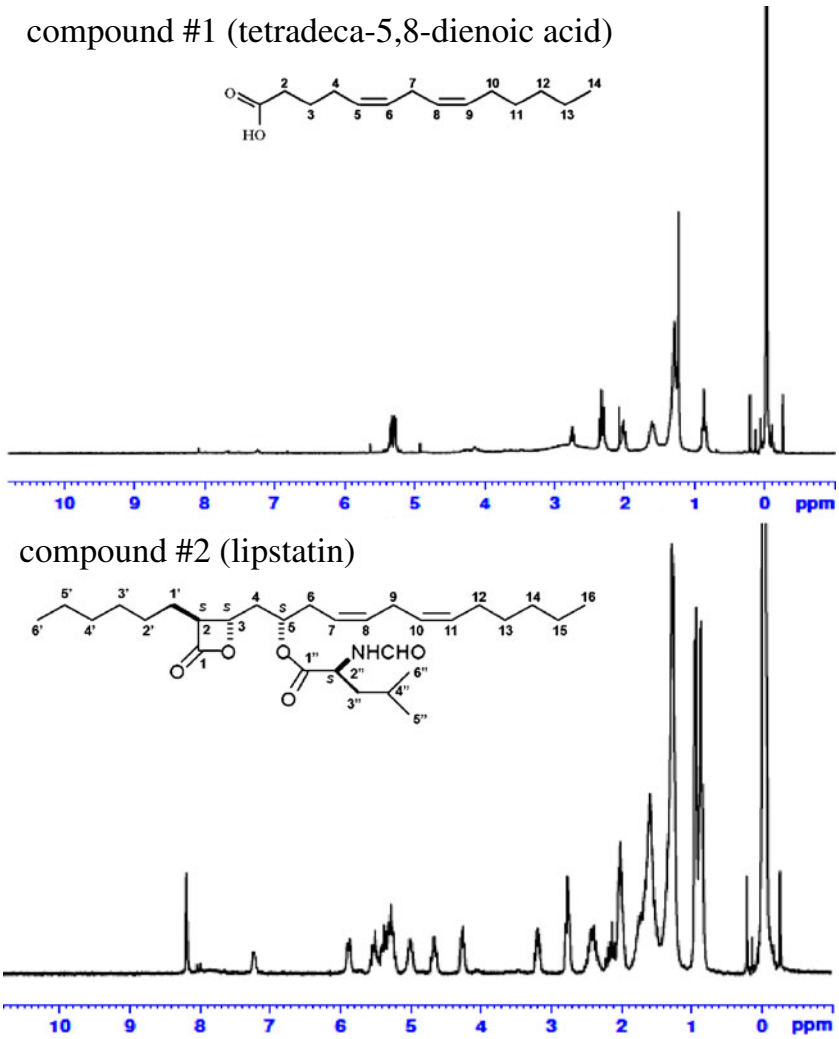

Fig. 7 ^{1}H–NMR spectrum of compound 1 (tetradeca-5,8-dienoic acid) from mutant strain and compound 2 (lipstatin) from wild-type strain isolated by preparative HPLC. Both compounds gave the proton shift at 5.5 ppm as multiplet for the unsaturated double bonds. In lipstatin, the protons in the lactone ring were shifted at 3.2 and 4.3 ppm as multiplets, and the characteristic proton shifts in the formylleucine residue also appeared at 8.2 ppm for 2″-amino group and at 5.9 ppm for the aldehyde group

clusters in two distinct DNA fragments of 11.2 kb (data not shown) and 10.6 kb from different locus of its genome. Sequence analysis of the first fragment revealed two genes, *accA1* and *accD1*, involved in primary metabolism (fatty acid biosynthesis) (Demirev et al. 2009). In contrast, the ten distinct ORFs identified in the second DNA fragment (10.6 kb) showed high homology with similar gene clusters in other *Streptomyces* spp. and are thought to be involved in secondary metabolism. The *pccE* and *accA3* genes, encoding ε and α subunits of ACCase, were present downstream of *pccB* gene encoding β subunit of ACCase.

In *S. coelicolor* and *M. tuberculosis*, ACCase consists of three subunits, the α, β, and ε subunit (Diacovich et al. 2002; Gago et al. 2006; Oh et al. 2006). The α subunit (BC) transfers CO_2 molecule to form a carboxyl–BC. Subsequently, the β subunit (CT) transfers the carboxyl group from carboxyl–BC to the acyl-CoA and controls the substrate specificity in each ACCase complex. A recent study showed that the gene products of *pccB* and *pccE* in *S. coelicolor* form a protein complex and PccB protein exhibits full activity only in presence of PccE subunit (Gago et al. 2006).

Based on the sequence homology with AccD5 and its putative activity, it was assumed that PccB is related to the secondary metabolism and probably recognizes and activates a fatty acid with longer chain than acetyl-CoA. In addition, the organization of 10.6 kb *pccB* gene locus showed that they could be functionally related to form a specific ACCase protein complex.

In order to disclose whether the gene products of *pccB* and *pccE* are involved in the lipstatin biosynthesis, these two genes were inactivated by homologous recombination in the wild-type *S. toxytricini* strain. The gene replacement by double-crossover in disruptant *S. toxytricini* BE-AP was confirmed by DNA hybridization and PCR amplification.

After the fermentative production of active metabolites, the final pH of the fermentation broth of the disruptant strain was lower than the wild-type, probably due to the accumulation of organic acids. There were no visible differences in growth between those strains after 4-day incubation. The metabolites in fermentation broths of wild-type and disruptant strain were extracted by hexane–ethyl acetate mixture, and it was observed that the spot corresponding to lipstatin in wild-type strain was reduced to a great extent in the disruptant in TLC analysis.

In the pancreatic lipase inhibitory assay, the crude extract of the disruptant exhibited considerably lower activity compared with that of the wild-type. However, the disruptant extract still inhibited the pancreatic lipase. Two metabolites purified from wild-type extract by preparative HPLC were subjected to ^{1}H-NMR for determination of their chemical structures. The ^{1}H-NMR spectrum of these compounds showed that they are lipstatin and tetradeca-5.8-dienoic acid, respectively. The HPLC chromatograms of extracts showed that the disruptant did not only accumulate the major precursor tetradeca-5.8-dienoic acid, but also produced a lower level of the active metabolite lipstatin.

Earlier tracer study experiments for elucidations of the specific precursors (Goese et al. 2001; Eisenreich et al. 2003; Schuhr et al. 2002) showed that Claisen condensation of 3-hydroxy-tetradeca-5.8-dienoic acid and octanoic acid leads to formation of the lipstatin backbone. Considering the crystal structure of PccB and its recognition of longer chain fatty acid than acetyl-CoA but shorter than linoleyl-CoA (Diacovich et al. 2004), it can be assumed that PccB of *S. toxytricini* is involved in the activation of octanoic acid to carboxyl–octanoic acid, which is the second major precursor in the lipstatin biosynthesis.

In addition, it can be speculated that the lipids were actively metabolized by *S. toxytricini* BE-AP strain to monoglycerides and linoleic acid, which was further metabolized by β–degradation to tetradeca-5.8-dienoic acid. But, the resulting fatty acid could not be utilized by disruptant for lipstatin biosynthesis because octanoic acid was not activated to hexylmalonic acid. However, the lipstatin biosynthesis was not completely turned off in disruptant, probably due to the activation of octanoic acid by another ACC complex system with much lower activity.

The accumulation of tetradeca-5.8-dienoic acid rather than 3-hydroxy-tetradeca-5.8-dienoic acid by disruptant suggests that the hydroxyl group can be introduced at C-5 position of the generated 3-oxo intermediate after the Claisen condensation. The accumulation of this compound also could be the reason for the lower pH after fermentation with the disruptant strain.

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