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Type II thioesterase ScoT is required for coelimycin production by the modular polyketide synthase Cpk of *Streptomyces coelicolor* A3(2)*

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Type II thioesterases were shown to maintain efficiency of modular type I polyketide synthases and nonribosomal peptide synthetases by removing acyl residues blocking extension modules. We found that thioesterase ScoT from *Streptomyces coelicolor* A3(2) is required for the production of the yellow-pigmented coelimycin by the modular polyketide synthase Cpk. No production of coelimycin was observed in cultures of *scoT* disruption mutant. Polyketide production was restored upon complementation with an intact copy of the *scoT* gene. An enzymatic assay showed that ScoT thioesterase can hydrolyse a 12-carbon acyl chain but the activity is too low to play a role in product release from the polyketide synthase. We conclude that ScoT is an editing enzyme necessary to maintain the activity of polyketide synthase Cpk. We provide a HPLC based method to measure the amount of coelimycin P2 in a culture medium.

Key words: type II thioesterase, coelimycin, coelimycin measurement, *Streptomyces coelicolor*, polyketide synthase, Cpk

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INTRODUCTION

Polyketides and non-ribosomal peptides form two large and diverse groups of natural compounds with a number of biological activities (antibiotics, immunomodulators, antifungal and anticancer agents). They are synthesized by several rounds of condensations of short building blocks (carboxylic acids in case of polyketides and aminoacids in case of peptides). Chain elongation is accomplished by multienzyme complexes called polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) (Staunton & Weissman, 2001; Koglin & Walsh, 2009). Hybrid multienzymes composed of both, PKS and NRPS parts, are also known (Boettger & Hertweck, 2013). Great attention has been given to modular type I PKS and NRPS systems due to their great potential for the synthesis of novel compounds by a combinatorial approach (Olano *et al.*, 2009; Wong & Khosla, 2012). These megasynthases are built of multifunctional proteins in which enzymatic domains required for each round of condensation and modification are structurally organized in modules which form an "assembly line". The growing chain and extender units are attached to the multienzyme as thioesters with phosphopantetheine arm of an acyl carrier protein (ACP) domain in PKS or peptidyl carrier protein (PCP) domain in NRPS. The chain is usually released by a thioesterase domain located at the C-terminus of the last module.

Genes coding discrete thioesterases (type II thioesterases, TEII) have been found within many PKS and NRPS gene clusters. Some of them are responsible for chain release (Oliynyk *et al.*, 2003; Liu *et al.*, 2006; Liu *et al.*, 2008), however most of TEIIs studied so far have been shown to have editing functions. These include control of starter unit selection (Hu *et al.*, 2003; Rui *et al.*, 2010; Kalaitzis *et al.*, 2011) and removal of non-reactive acyl residues attached to phosphopantetheine which block the entire "assembly line" (Heathcote *et al.*, 2001; Kim *et al.*, 2002; Schwarzer *et al.*, 2002; Yeh *et al.*, 2004).

In the modular type I polyketide synthase Cpk from *Streptomyces coelicolor* A3(2) a reductase domain is located at the C-terminus of the last module instead of a typical thioesterase domain (Pawlik *et al.*, 2007). This terminal thioester reductase was proposed to release the product of polyketide synthase Cpk as a putative hydroxyaldehyde which is further transformed into a putative antibacterial bis-epoxide coelimycin A. Non-enzymatic reactions of the epoxide rings with compounds present in the culture medium give rise to yellow-pigmented coelimycin P1, coelimycin P2 and possibly other related products, some of which may have no visible colour (Gomez-Escribano *et al.*, 2012; Challis 2013).

The *cpk* gene cluster contains the *scoT* gene coding a type II thioesterase. In previous studies we have shown that ScoT can functionally replace TylO, an editing thioesterase accompanying tylosin producing modular PKS in *Streptomyces fradiae* (Kotowska *et al.*, 2002). Recombinant ScoT protein can hydrolyse acetyl, propionyl and butyryl residues, with preference for propionyl, which is consistent with its proposed role as an editing enzyme (Kotowska *et al.*, 2009). Analysis of ScoT amino acid sequence with model building server Phyre 2 (Kelley & Sternberg, 2009) showed the highest similarity of predicted structure to editing type II thioesterase RifR from a hybrid PKS-NRPS rifamycin biosynthetic cluster of *Amycolatopsis mediterranei* (Claxton *et al.*, 2009). ScoT and

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Abbreviations: ACP, acyl carrier protein; NRPS, non-ribosomal peptide synthetase; PCP, peptidyl carrier protein; PKS, polyketide synthase; TEII, type II thioesterase

Table 1. Strains of *Streptomyces coelicolor* A3(2) and plasmids used in this study

Strain	Description	Reference
M145	SCP1$^-$, SCP2$^-$	Kieser *et al.*, 2000
MK120	derivative of M145 with insertional disruption of *scoT* gene (*scoT::hyg*)	this work
MK121	strain with disruption of *scoT* complemented by *scoT* gene under *ermEp** promoter (*scoT::hyg* + *scoT*)	this work
MK122	strain for control of complementation (*scoT::hyg*/pLST9828)	this work
P100	derivative of M145 with disruption of *cpkC* gene	Pawlik *et al*., 2007
Plasmid	**Description**	**Reference**
pTEinshyg	derivative of pOJ260 containing hygromycin resistance cassette Ω*hyg* between flanking arms TE-1 and TE-2; used for *scoT* gene disruption	this work
pLST9828-scoT	derivative of pLST9828 containing *scoT* gene used for complementation	Kotowska *et al*., 2002
pLST9828	vector for conjugal transfer from *E. coli* to *Streptomyces*, integrates into ΦC31 *attB* site, Apra^R	Butler *et al*., 1999
pOJ260	vector for conjugal transfer from *E. coli* to *Streptomyces*, non-integrative, Apra^R, does not propagate in *Streptomyces*	Bierman *et al.*, 1992
pIJ2529-hyg	provides hygromycin resistance cassette Ω*hyg* (Blondelet-Rouault *et al*., 1997) as BamHI-BamHI fragment	gift from A. Butler, University of Leicester, UK

RifR have 52% identical and 64% similar amino acids as shown by blastp comparison.

Discovery of the yellow-pigmented coelimycin which is the product of polyketide synthase Cpk and establishing growth conditions required to obtain its production in wild type strain *S. coelicolor* M145 (Pawlik *et al*., 2010) enabled us to study the role of ScoT in its native host. The aim of the current work was to investigate the role of ScoT thioesterase with respect to production of coelimycin by Cpk synthase. Moreover, we present here a method of quantification of coelimycin P2 in a culture medium.

MATERIALS AND METHODS

Plasmids, bacterial strains and culture conditions. *Streptomyces* strains and plasmids used in this study are listed in Table 1. *Streptomyces* strains were grown on MS agar for sporulation and mating (Kieser *et al*., 2000). Polyketide (yellow pigment) production was observed on modified 79 medium (Pawlik *et al*., 2010), Glu-DNA (Gottelt *et al*., 2010), Glu-MM (minimal medium prepared according to Kieser *et al*. (2000) supplemented with 50 mM sodium glutamate instead of asparagine). For HPLC analysis, 25 ml cultures were grown in 250 ml Erlenmeyer flasks containing stainless steel springs (Kieser *et al*., 2000). Glu-MM liquid medium was inoculated with spore suspensions to equal optical density for each strain (OD_{600} = 0.2) and shaken at 30°C on a reciprocating shaker. Samples were taken after 24 hours of growth, centrifuged (5 min, 15 000 × *g*) and used for HPLC analysis directly or after freezing at –20°C. Cultures were run in triplicate.

E. coli ET12567 /pUZ8002 was used to introduce DNA into *S. coelicolor* via intergeneric conjugal transfer (Kieser *et al*., 2000). *E. coli* DH5α was used for routine plasmid manipulations and was grown on LB media (Sambrook *et al*., 2001).

DNA manipulations. Standard methods were used for DNA manipulations (Sambrook *et al*., 2001). Isolation of genomic DNA from *Streptomyces* strains was carried out using standard protocols (Kieser *et al*., 2000). Digoxin DNA labeling and Southern blot analyses were performed according to "The DIG System User's Guide for Filter Hybridization" (Boehringer Mannheim). CSPD was used as chemiluminescent substrate for detection. All PCR amplified fragments were first cloned into pGEM-T Easy vector (Promega) to facilitate their digestion with restriction enzymes and further cloning into appropriate plasmids. Primers used are listed in Table 2.

Disruption of the *scoT* gene. The *scoT* gene (SCO6287, number according to Bentley *et al*., 2002) was disrupted by insertion of hygromycin resistance gene in the process of homologous recombination. Flanking arms were amplified from cosmid St1G7 (http://strepdb.streptomyces.org.uk) with the following primer pairs: scbR2-Rev and TE-1Rev (flanking arm TE-1), TE-2Fw and TE-2Rev (flanking arm TE-2). The arms were cloned sequentially into pOJ260 vector (TE-1 into HindIII and XbaI sites, TE-2 into XbaI and EcoRI sites). Hygromycin resistance cassette Ω*hyg* (Blondelet-Rouault

Table 2. Primers used in this study. Restriction sites are underlined.

Primer	Nucleotide sequence (5′-3′)	Restriction site, purpose
ScbR2-Rev	TTTTTTAAGCTTAGCTGGCGGAACGCGGCG	*Hin*dIII, cloning of flanking arm TE-1
TE-1Rev	AAAAAATCTAGAACCAGTCACTTCCC	*Xba*I, cloning of flanking arm TE-1
TE-2Fw	AAAAAATCTAGAGGCGGTTCGGTCCCG	*Xba*I, cloning of flanking arm TE-2
TE-2Rev	AAAAAAGAATTCGTTCGGCCGGGTCAG	*EcoR*I, cloning of flanking arm TE-2
att_F	GGGCAGGGTAACCTCGGCGCC	amplification of probe *attB*
att_R	AACGGCCTGAAGGAGCTGCGCC	amplification of probe *attB*

et al., 1997) obtained as BamHI restriction fragment from pIJ2925-hyg plasmid was cloned into XbaI site between the two flanking arms by blunt end ligation after filling of overhangs with the Klenow enzyme, yielding plasmid pTEinshyg. Plasmid pTEinshyg was introduced into *S. coelicolor* M145 *via* conjugation. Transconjugants were screened for hygromycin resistance and apramycin sensitivity as indication of a double *cross-over* event. Obtained disruption mutant strain was named *S. coelicolor* MK120. Gene disruption was confirmed by Southern hybridization. BamHI digested chromosomal DNA of M145 and MK120 strains was probed with digoxigenin labeled BamHI fragment "6" (1385 bp) covering N-terminal part of the *scoT* gene together with upstream sequence (Kotowska et al., 2002).

Complementation of the *scoT* disrupted strain. Construction of a derivative of pLST9828 carrying the *scoT* gene under a strong constitutive promoter *ermE*p* was described earlier (Kotowska *et al.*, 2002). Plasmid pLST9828-scoT was introduced by conjugation into MK120 yielding complemented strain MK121. In parallel, control strain MK122 was prepared by introduction of an empty plasmid pLST9828 into MK120. Integration into chromosomal attachment site was confirmed by Southern blot. NcoI digested chromosomal DNA of M145, MK121 and MK122 was hybridized with DIG-labeled *attB* probe obtained as a PCR product with primers attF and attR. Probe *attB* covers 2248 bp surrounding chromosomal attachment site (*attB*) of phage ΦC31.

HPLC analysis of coelimycin P2 production. Dionex 3000 HPLC system equipped with LPG-3400SD pump, WPS-3000T(B) FC Analytical autosampler, TCC-3000SD column compartment, DAD-3000 diode array detector and FLD-3400RS 2 PMT fluorescence detector (Dionex Corporation) was used. The HPLC separation was performed using a reverse-phase Durashell C18 column, 250 × 4.6 mm, 5-μm particle size (Agela Technologies). The autosampler vials temperature was set at 8°C and the column temperature was set at 22°C. The mobile phase: solvent A consisted of methanol (5%), formic acid (0.1%) and water (95%); solvent B consisted of methanol (95%), formic acid (0.1%) and water (5%). Volume of injected sample was 50 μl. The elution profile was as follows: 100% solvent A to 100% solvent B for 15.1 minutes, then isocratic at 100% solvent B for 1 minute, back to 100% solvent A for 0.4 minutes and equilibrated for 6

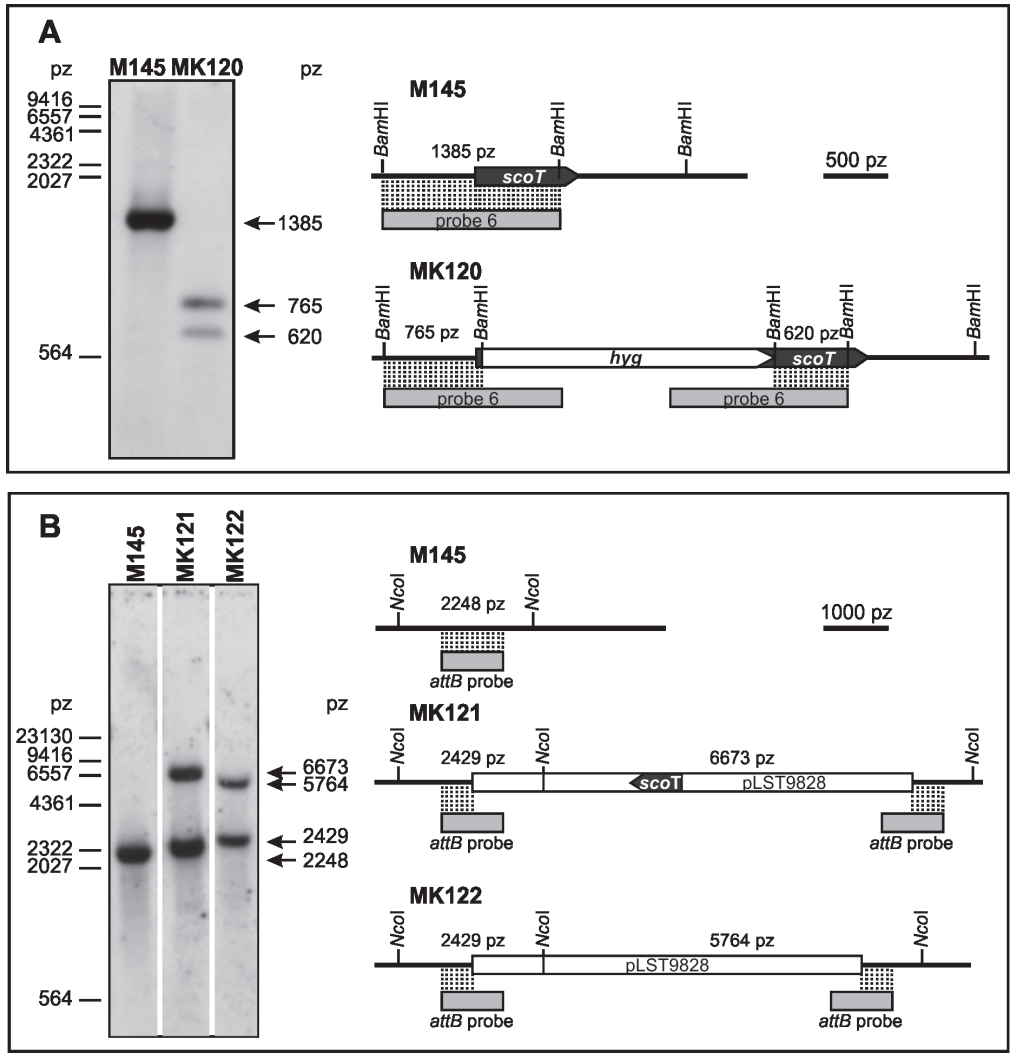

Figure 1. Southern blot analysis of strains constructed in this study.
(**A**) Hybridization of BamHI digested chromosomal DNA with probe "6". (**B**) Hybridization of NcoI digested chromosomal DNA with probe *attB*. M145 — wild type strain, MK120 — *scoT* disruption, MK121 — complementation with intact *scoT* gene, MK122 — control of complementation (empty plasmid in MK121). Regions complementary to probes are marked with dotted lines. Hybridized fragments of expected sizes are marked with arrows. Molecular weight marker used was λ DNA digested with HindIII.

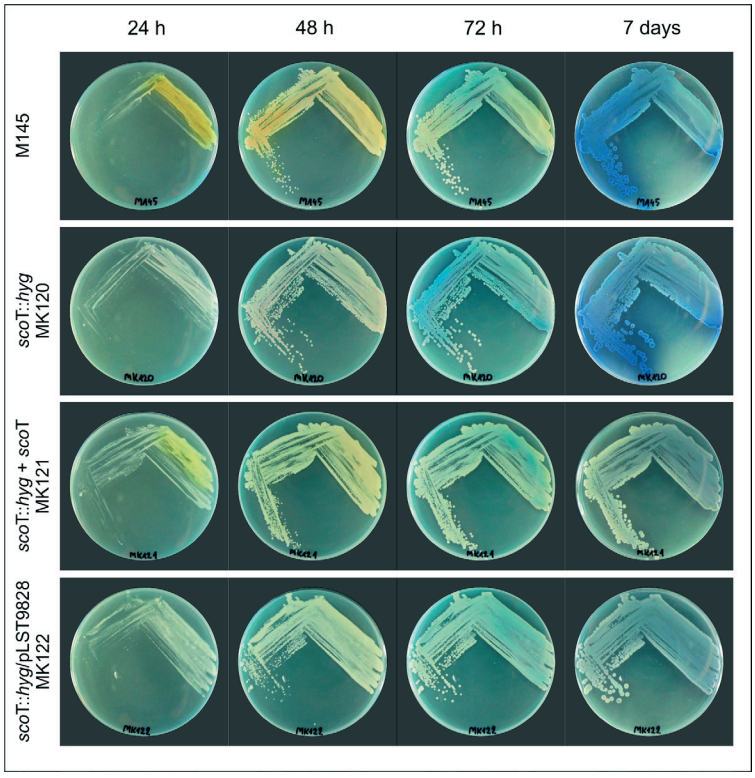

Figure 2. Growth of *S. coelicolor* A3(2) strains on modified medium79.
Coelimycin is visible as a yellow pigment diffusing to the medium. M145 — wild type strain, MK120 — *scoT* disruption, MK121 — complementation with intact *scoT* gene, MK122 — control of complementation (empty plasmid in MK121).

minutes before the next injection. The flow rate was 1 ml/minute. The absorbance detection was performed at a wavelength of 427 nm.

Assay of enzymatic activity of thioesterase. ScoT recombinant protein was purified as described earlier (Kotowska *et al.*, 2009). Tested substrates were *p*-nitrophenyl dodecanoate and *p*-nitrophenyl propionate. Substrate stock solutions were 3 mM in DMSO. Hydrolysis was visible due to formation of yellow *p*-nitro-phenolate anion and was monitored by spectroscopy (λ = 400 nm; ε = 8570 $M^{-1}cm^{-1}$). Hydrolysis of propionate was monitored for 3 minutes. Hydrolysis of dodecanoate was measured after 220 minutes. Reactions were run at 30°C in 1 ml (total volume) samples containing 200 mM potassium phosphate (pH 7.5), 3% (v/v) DMSO, 90 μM substrate, and variable concentrations of protein: 0.15 μM for hydrolysis of propionate and 2.9, 4.3 and 5.8 μM for dodecanoate. The results were corrected for background hydrolysis in the absence of the enzyme.

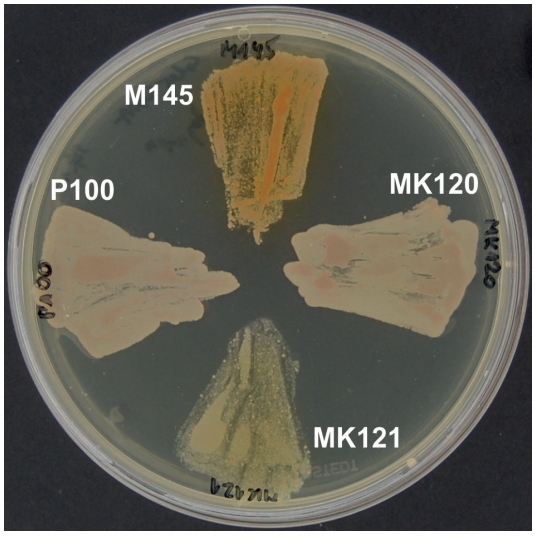

Figure 3. Growth of *S. coelicolor* A3(2) strains on Glu-DNA medium.
Coelimycin is visible as a yellow pigment diffusing to the medium. M145 — wild type strain, MK120 — *scoT* disruption, MK121 — complementation with intact *scoT* gene, P100 — strain with inactivated *cpkC* gene unable to produce coelimycin.

RESULTS

Effect of thioesterase ScoT deletion on coelimycin production

In order to analyze the role of type II thioesterase from *cpk* cluster, *scoT* gene was disrupted by insertion of hygromycin resistance cassette into *S. coelicolor* M145 chromosome. Gene disruption was confirmed by Southern hybridization (Fig. 1). No production of the yellow pigment by *S. coelicolor* MK120 strain (disruption mutant) was observed on media known to promote coelimycin synthesis: modified 79 medium (Fig. 2), Glu-DNA (Fig. 3), DNA and Glu-MM (Fig. 4, Table 3). The phenotype was identical to that of strain P100 (Pawlik *et al.*, 2010) which cannot produce coelimycin due to disruption of one of the main PKS subunits (Fig. 3). To ensure that the observed phenotype of MK120 was solely due to disruption of the thioesterase gene, and not a polar effect, an intact copy of *scoT* was supplied on an integrative vector. Integration was confirmed by Southern blot (Fig. 1). Complementation restored the yellow pigment production to wild type level (Figs. 2–4, Table 3).

Culture supernatants of all strains grown in Glu-MM liquid medium were analyzed by means of HPLC as described in Materials and Methods (Fig. 4). A single peak which eluted at 8.08 min detected in the wild type strain was identified as coelimycin P2 (a glutamate adduct of coelimycin A) by mass spectrometry on Bruker micrOTOF-QII instrument (calculated m/z 351.1551, found 351.1558). The mass is in agreement with the chemical formula of this compound as proposed by Gomez-Escribano *et al.* (2012).

Table 3. Production of coelimycin P2 by *S. coelicolor* A3(2) strains in 24 h Glu-MM liquid cultures measured as area under a peak eluted at 8.08 min. HPLC separation method is described in Materials and Methods.
The data are the means ± standard deviations.

strain	peak area	% of wild type
M145	14.65±0.19	100
MK120	0	0
MK121	13.79±1.68	94
MK122	0	0
P100	0	0

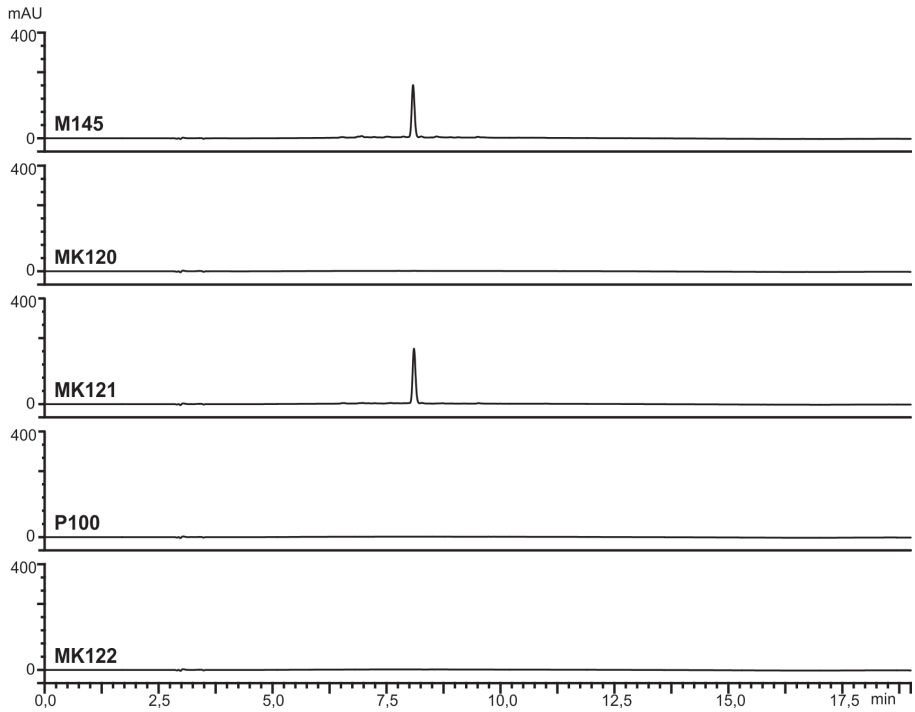

Figure 4. Chromatograms from HPLC analyses monitoring absorbance at 427 nm of culture supernatants of *S. coelicolor* A3(2) strains grown in Glu-MM liquid medium.
The 8.08 min peak was confirmed by mass spectrometry as coelimycin P2. The peak areas are compared in Table 3. M145 — wild type strain, MK120 — *scoT* disruption, MK121 — complementation with intact *scoT* gene, MK122 — control of complementation (empty plasmid in MK121) P100 — strain with inactivated *cpkC* gene unable to produce coelimycin.

Hydrolytic activity of ScoT thioesterase towards a 12-carbon chain

Intrigued by the total inhibition of coelimycin production as a result of *scoT* deletion, we raised the question if thioesterase ScoT could have an additional role as an enzyme responsible for polyketide chain release. We tested its hydrolytic activity against *p*-nitrophenyl-dodecanoate, a substrate chosen to mimic a 12-carbon polyketide chain attached to the ACP domain of the last module of Cpk synthase. We found that recombinant protein ScoT is able to hydrolyze dodecanoate (Fig. 5), however much less efficiently than short chain acyl esters (Kotowska *et al.*, 2009). Specific rates of hydrolysis of *p*-nitrophenyl dodecanoate and propionate were 0.012 and 90.5 ((μM of substrate) min^{-1} (μM of enzyme)$^{-1}$), respectively.

DISCUSSION

In the current study we have shown that disruption of type II thioesterase gene *scoT* from Cpk gene cluster resulted in a complete loss of coelimycin production. This is similar to previous reports where type II thioesterase knock-out severely reduced the level of corresponding compounds.

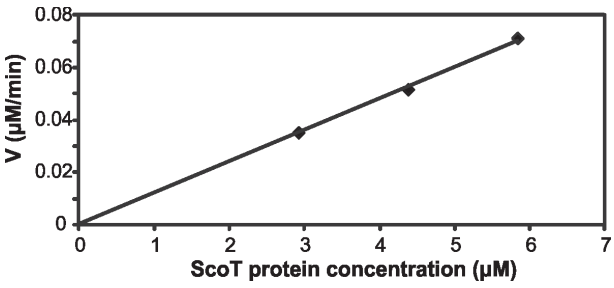

Figure 5. Rate of hydrolysis of *p*-nitrophenyl-dodecanoate by recombinant ScoT protein.
Substrate concentration was 0.09 mM.

Total inhibition of compound production is a typical effect of deletion of chain releasing type II thioesterases, while inactivation of TEIIs which have editing functions usually results in moderate decrease of product yield.

For example, deletion of *monCII* and *nanE* genes coding discrete thioesterases involved in polyether ionophore antibiotics synthesis completely blocked production of monensin and nanchangmycin, respectively (Oliynyk *et al.*, 2003; Liu *et al.*, 2006). Similarly, disruption of TEII associated with the cluster of streptolydigin from *Streptomyces lydicus* abolished its production (Yu *et al.*, 2006). Both monensin and nanchangmycin synthases lack C-terminal TE domains, and hydrolysis of the respective final products by MonCII and NanE was confirmed experimentally (Oliynyk *et al.*, 2003; Liu *et al.*, 2006; Liu *et al.*, 2008). Streptolydigin synthesis pathway by a hybrid PKS-NRPS system (both multienzymes lacking TE domains) was recently proposed, but the authors made no comments about the mode of chain release (Gomez *et al.*, 2012).

Effects of inactivation of type II thioesterases thought to have editing functions differ considerably. Some megasynthases seem to function well even in the absence of TEIIs (Chen *et al.*, 2000; Hu *et al.*, 2003). On the other hand, *rif*R deletion in *Amycolatopsis mediterranei* lowered rifamycin B production to 40–60% of normal levels (Doi-Katayama *et al.*, 2000), in case of *tylO* mutant of *S. fradiae*, tylosin production dropped to 15% (Butler *et al.*, 1999), while disruption of TEII associated with the cluster of a linear polyketide ECO-0501 (LW01) from *Amycolatopsis orientalis* reduced LW01 production by 95% (Shen *et al.*, 2012). LW01 production was restored by

complementation with a heterologous TEII PikAV from picromycin cluster (Shen *et al*., 2012) which was shown earlier to act as an editing thioesterase (Kim *et al*., 2002).

Our previous studies strongly supported the editing role of ScoT thioesterase. It was found to be active *in vitro* towards substrates which mimic short acyl residues blocking the polyketide "assembly line" (Kotowska *et al*., 2009). Moreover, the *scoT* gene was able to complement *tylO* disruption in *S. fradiae* (Kotowska *et al*., 2002). Here, we report a crucial role of ScoT thioesterase for its native polyketide synthase and provide evidence, that this enzyme is necessary for coelimycin biosynthesis. This is an unusual feature of an editing thioesterase. Therefore, a hypothesis of a potential chain terminating activity of ScoT was tested.

It was shown that the ScoT protein can hydrolyse *p*-nitrophenyl dodecanoate. Enzymatic assay showed that the reaction rate for this compound is approximately 7500 times lower than for its preferred substrate — propionate. Therefore, the hypothesis that thioesterase ScoT could release the final polyketide chain from Cpk synthase seems unlikely. Such a slow rate of product hydrolysis would be a limiting step in biosynthesis and would facilitate hydrolysis of shorter intermediates attached to preceding modules by the same thioesterase. However, this result indicates that ScoT thioesterase has a broad range of substrates and may also digest longer acyl chains which are exposed to its action for a sufficiently long period of time. We realize that the use of p-NF derivatives as model substrates has its drawbacks but it allows to compare enzyme preference for substrates of different chain length. A substrate more precisely mimicking the natural situation would require synthesis of the putative 4-hydroxy-2,6,8,10-dodecatetraenoyl thioester attached to the ACP from the 5th module or to the whole CpkC protein, as other factors related to the whole Cpk synthase could be important. Also, investigation of the enzymatic activity of the terminal thioester reductase domain would shed more light on the mechanism of release of coelimycin precursor from the polyketide synthase.

It is not clear why some megasynthases apparently do not need type II thioesterases while the activity of others strongly depends on the presence of the editing enzymes. Our results suggest that Cpk synthase is exceptionally sensitive to blocking by non-reactive residues. Two main mechanisms were proposed which lead to synthase blocking by non-reactive acyl residues: aberrant decarboxylation of extender units ((Heathcote *et al*., 2001; Kim *et al*., 2002) and ACP/PCP mispriming with acyl phosphopantetheine (Schwarzer *et al*., 2002). These processes are poorly understood. Clarification of reasons for differences in sensitivity of megasynthases to blocking requires detailed structural studies of whole synthase complexes.

We have reported here a method of detection and relative quantification of coelimycin P2 based on HPLC analysis. The use of the minimal medium instead of rich media such as 79, DNA, R3 (Pawlik *et al*., 2010; Gottelt *et al*., 2010; Gomez-Escribano *et al*., 2012) allowed to obtain a simple chromatogram in a direct measurement of culture medium of wild type strain. Thanks to supplementation with glutamate, the double epoxide (coelimycin A) is transformed mainly into coelimycin P2, as opposed to a mixture of compounds detected in the culture of *S.coelicolor* M145 in R3 medium (not shown).

In conclusion, we have shown that type II thioesterase ScoT is crucial for coelimycin production by *Streptomyces coelicolor* A3(2) and provided a method to detect coelimycin P2 in a culture medium. To our knowledge, this is currently the most effective method available for measurement of the activity of Cpk synthase.

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