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# Type II thioesterase from *Streptomyces coelicolor* A3(2)

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**Type I polyketide synthases (PKSs) are complexes of large, multimodular enzymes that catalyse biosynthesis of polyketide compounds via repetitive reaction sequences, during which each step is catalysed by a separate enzymic domain. Many type I PKSs, and also non-ribosomal peptide synthetase clusters, contain additional thioesterase genes located adjacent to PKS genes. These are discrete proteins called type II thioesterases (TE IIs) to distinguish them from chain-terminating thioesterase (TE I) domains that are usually fused to the terminal PKS module. A gene of a new TE II, *scoT*, associated with the cluster of putative type I PKS genes from *Streptomyces coelicolor* A3(2), was found. The deduced amino acid sequence of the gene product shows extensive similarity to other authentic thioesterase enzymes, including conservation of characteristic motifs and residues involved in catalysis. When expressed in the heterologous host *Streptomyces fradiae*, *scoT* successfully complemented the resident TE II gene (*tylO*), and, by restoring a significant level of macrolide production, proved to be catalytically equivalent to the TylO protein. $S_1$ nuclease mapping of *scoT* revealed a single potential transcription start point with expression being switched on for a short period of time during a transition phase of growth.**

**Keywords:** thioesterase type II, *Streptomyces fradiae*, disruption mutant complementation, $S_1$ nuclease mapping

## INTRODUCTION

Polyketides are a large and structurally a diverse group of compounds, many of which exhibit biological activity as antibiotics, immunosuppressants or anticancer drugs. Polyketides are synthesized by a common mode of condensation and reduction reactions similar to that of fatty acid biosynthesis. Nascent polyketides are processed by large multienzyme complexes, polyketide synthases (PKSs). In the type I PKSs, involved in production of macrolide antibiotics such as erythromycin, reactions of the biosynthetic cycle are catalysed sequentially by separate enzymic domains housed in large multifunctional polypeptides. Each complete cycle of condensation and reduction reactions is catalysed by a module, a functional unit of the PKS. The substrate acyl chains which undergo successive reactions are tethered as thioesters by acyl carrier domains of the PKS polypeptides. A terminal thioesterase domain (TE) catalyses release and cyclization of the full-length (fully processed) polyketide chain (Katz & Donadio, 1993).

Many type I PKSs, and also non-ribosomal peptide synthetase clusters, contain additional TE genes located adjacent to the PKS genes within the cluster of antibiotic biosynthetic genes (Weissman *et al*., 1998; Schneider & Marahiel, 1998; Shaw-Reid *et al*., 1999; August *et al*., 1998; Xue *et al*., 1998; Heathcote *et al*., 2001). The products of such genes are discrete proteins called type II thioesterases (TE IIs) to distinguish them from chain-terminating thioesterase (TE I) domains (Gokhale *et al*., 1999). In fatty acid synthase complexes, TE IIs are alternative chain-terminating enzymes exhibiting hydrolase activity towards medium-chain-length acyl thioesters (Smith, 1994). There is no obvious role for the TE IIs associated with multienzyme PKS complexes, nor is the mechanism of their action known.

**Abbreviations:** PKS, polyketide synthase; TE, thioesterase.

The GenBank accession number for the sequence reported in this paper is AF109727.

**Table 1.** *Streptomyces* strains used in this work

| Strain | Genotype | Reference |
|---|---|---|
| *S. coelicolor* A3(2) M145 | SCP1$^-$ SCP2$^-$ | Hopwood *et al.* (1985) |
| *S. fradiae* C373.1 | Wild-type, tylosin producer | Seno & Baltz (1982) |
| *S. fradiae* C373.1, disruption mutant strain | Δ*tylO* | Butler *et al.* (1999) |
| *S. fradiae* C373.1, disruption mutant strain, complemented | Δ*tylO scoT* | This study |

The function of TE IIs is predicted from gene-disruption analysis, complementation studies, and determination of their substrate specificities (Weissman *et al.*, 1998; Butler *et al.*, 1999; Heathcote *et al.*, 2001). Polyketide production is drastically reduced, by 90% or more, in strains with a deleted TE II gene (Xue *et al.*, 1998; Butler *et al.*, 1999; Doi-Katayama *et al.*, 2000), indicating an important function, proposed to involve editing of aberrant intermediates (Butler *et al.*, 1999) during the course of polyketide biosynthesis. More recently, this hypothesis has been confirmed and the mechanism clarified. Thus, the TylO protein displayed hydrolytic activity *in vitro* towards short-chain acyl-CoAs, indicating that the enzyme could remove aberrantly decarboxylated (and, therefore, non-reactive) extender acyl chains from the PKS during polyketide biosynthesis (Heathcote *et al.*, 2001). By hydrolytic release of such aberrant acyl groups, TE II was proposed to unblock PKS modules and restore overall efficiency of the complex enzyme.

The proposed common role for TE IIs in PKS multienzyme systems raises the question of whether specific TE IIs might be replaceable by other TE IIs normally associated with other PKS complexes. Based on the TE II substrate-specificity studies published to date (Weissman *et al.*, 1998; Heathcote *et al.*, 2001), such enzymes do not seem to select the structure of the acyl substrates.

Genetic engineering studies allow assembly of novel polyketide chains following fusion, swapping or repositioning of catalytic domains, modules or whole peptides within PKS polypeptides (Hutchinson & Fujii, 1995; Ranganathan *et al.*, 1999; Tang *et al.*, 2000). In engineered PKSs, co-expression of TE IIs in addition to other PKS proteins might help in achieving elevated levels of the polyketide products.

We were studying a gene cluster for the new PKS type I in *Streptomyces coelicolor* A3(2) (Kuczek *et al.*, 1997; K. Pawlik, M. Kotowska & K. Kuczek, unpublished data; GenBank accession numbers U88833, AF109727 and AF 202898) located in a previously unmapped region of the chromosome, between cosmids 2H4 and 10H5 (Redenbach *et al.*, 1996), now covered by cosmids 2C4, 1G7, BAC8D1 and IF3 (http://www.sanger.ac.uk/Projects/S_coelicolor/). During our studies, we found a new gene, encoding a putative TE II (ScoT). The gene is located within the putative polyketide biosynthetic gene cluster. In this paper, we describe this gene. When expressed in the heterologous host, *Streptomyces fradiae*, *scoT* functionally complemented disruption of the native TE II gene, *tylO*.

## METHODS

**Bacterial strains, cosmid clones, plasmids and growth media.** The *Streptomyces* strains used are listed in Table 1. *S. fradiae* and its mutants were maintained and propagated at 37 °C on AS1 agar (Wilson & Cundliffe, 1998) or at 30 °C in Difco tryptic soy broth. *S. coelicolor* A3(2) was maintained and propagated at 30 °C on 79 agar medium [IMET Catalogue of Strains (Jena) 1987] or SMM agar (Kieser *et al.*, 2000). *Escherichia coli* SURE strain [e14$^-$(McrA$^-$) Δ(*mcrCB–hsdSMR–mrr*)*171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC*::Tn5 (Kan$^r$) *uvrC* (F′ *proAB lacI*$^q$*Z* ΔM15 Tn*10* (Tet$^r$)] was a host for cosmid clone 1G7 of *S. coelicolor* A3(2) genomic DNA in SuperCos vector (Stratagene), which was kindly provided by H. M. Kieser (John Innes Institute, Norwich, UK). pBluescript SK(+) and SK(−) phagemid vectors (Stratagene) were used for cloning of restriction fragments from the cosmid and for their sequencing. *E. coli* K-12 strain DH5 α was used for routine plasmid manipulation and was grown in standard media (Sambrook *et al.*, 1989). DNA was introduced into *S. fradiae* via conjugal transfer from *E. coli* S17-1 (Simon *et al.*, 1983).

**DNA and RNA isolation, hybridization and sequencing.** Plasmids propagated in *E. coli* were isolated according to standard procedures (Sambrook *et al.*, 1989). Cosmid DNA was purified by the procedure for high-molecular-mass plasmid DNA preparation and was used for restriction analysis and cloning of *scoT* by standard genetic procedures (Sambrook *et al.*, 1989). The hybridization probe was random-prime labelled with DIG-11-dUTP. Labelling, hybridization (at 68 °C) and detection of hybrids were carried out according to the manufacturer's recommended procedure (*The DIG System User's Guide for Filter Hybridization*; Boehringer) with CSPD (Tropix) as a substrate for the luminescent reaction. The sequence of the *S. coelicolor* DNA was determined both manually (Kuczek *et al.*, 1998), by using the chain-termination method with the Sequenase version 2.0 sequencing kit of Amersham, and by automated sequencing (performed by Qiagen Sequencing Services, Hilden, Germany). The sequence was determined on both strands and was submitted to GenBank (accession no. AF109727). Comparisons of the nucleotide and amino acid sequences with the databases were performed with the BLAST and CLUSTAL W programs. RNA was isolated as described by Strauch *et al.* (1991).

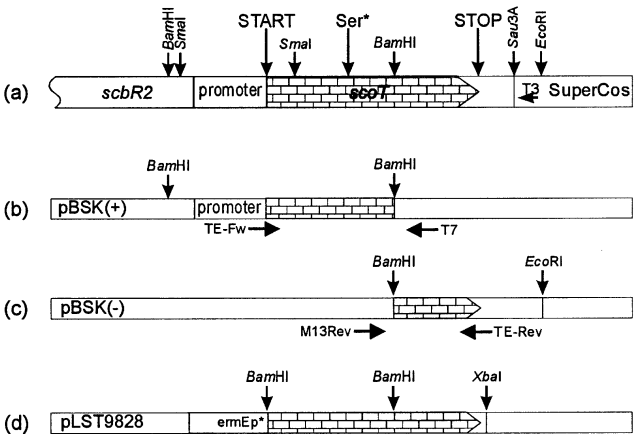


**Fig. 1.** Cloning strategy of *scoT* and its fragments in different vectors. (a) Restriction map of the T3-terminal fragment of cosmid 1G7 containing the *scoT* gene; *scbR2* denotes another ORF identified upstream of *scoT*. (b, c) Clones containing fragments of *scoT* encoding the N- and C-terminal parts of TE II, respectively. Arrows indicate primers used for PCR amplification of the respective fragments for cloning into the pLST9828 vector. (d) The recombinant plasmid pLST9828 containing the entire sequence of *scoT* for complementation study.

**Construction of a recombinant plasmid for complementation analysis.** An *S. fradiae tylO*-disruption strain was complemented with cloned *S. coelicolor* A3(2) DNA containing *scoT*, using the conjugative vector pLST9828 (Butler *et al.*, 1999). This is a derivative of pSET152 (Bierman *et al.*, 1992) that contains the powerful constitutive promoter *ermEp** to drive expression of inserted DNA fragments following integration into the chromosomal $\phi$C31 *attB* site. A DNA fragment containing *scoT* was ligated into pLST9828 in a two-step process. First, the DNA containing the C-terminal part of ScoT was PCR-amplified from the pBSK(−) plasmid clone template (Fig. 1c) using M13 reverse primer and the TE-Rev primer with an engineered *Xba*I site (underlined): 5′-TTTTCTAGATGTCGTACGTACACGGA-3′. The PCR product was purified using the Qiaex II DNA purification kit, digested, and ligated into pLST9828 using the *Bam*HI and *Xba*I sites. Then, a second PCR product containing the N-terminal part of ScoT was obtained from the template of another pBSK(+) construct (Fig. 1b) using the T7 universal primer and the TE-Fw primer with an engineered *Bam*HI site (underlined): 5′-TTTTTTGGATCCGATGGGAAGTGACTGGTT-3′. The 50 µl reaction mixture contained 5 µl 10 × PCR DyNAzyme buffer (Finnzymes), 1 µl 10 mM deoxynucleoside triphosphate mixture, 50 pmol each oligonucleotide, about 10 ng template DNA and 1 µl DyNAzyme II DNA polymerase (Finnzymes). Cycling was as follows: a hot start at 96 °C for 6 min, 1 min at 80 °C (adding of the enzyme), 31 cycles with denaturation at 95 °C for 1 min, annealing at 63–65 °C for 1 min and extension at 72 °C for 1·5 min, followed by a final extension at 72 °C for 5 min. The product was digested with *Bam*HI and ligated into the pLST9828 derivative obtained in the first step of the cloning procedure. Gentamicin (15 µg ml$^{-1}$) was used for the selection of *E. coli* DH5$\alpha$ transformants. The authenticity and orientation of the cloned fragments were confirmed by automated sequence analysis. The gene cloned in pLST9828 was introduced into *S. fradiae* C373.1, a tylosin-producing strain, by transconjugation from *E. coli* S17-1 as described elsewhere (Butler *et al.*, 1999).

**Fermentation and analysis of its products.** Strains of *S. fradiae* were fermented in media described elsewhere, and fermentation products were extracted and analysed by reverse-phase HPLC, with absorbance measurement at 282 nm, also as described elsewhere (Butler *et al.*, 1999). Desmycosin was used as an internal standard to identify fermentation products in *tylO*-disrupted strains.

**$S_1$ nuclease mapping.** For each $S_1$ nuclease reaction, 30 or 40 µg RNA was hybridized in NaTCA buffer [Murray, 1986; solid NaTCA (Aldrich) dissolved to 3 M final concentration in 50 mM PIPES, 5 mM EDTA, pH 7·0) to about 0·002 pmol (approx. $10^4$ Cerenkov c.p.m.] of the probe. The oligonucleotide 5′-GTCGAACTCGGGCGTCAGCTC-3′ was uniquely labelled at its 5′ end with [$^{32}$P]ATP using T4 polynucleotide kinase, and was used in the PCR with the unlabelled oligonucleotide 5′-CCTCGGCGGCGGAGAGAAT-3′, which anneals upstream of the *scoT* promoter, to generate a 430 bp probe. The PCR used M145 total DNA as a template. Subsequent steps were as described by Strauch *et al.* (1991).

## RESULTS

### TE II gene *scoT*

We investigated cosmid 1G7 from the library of chromosomal DNA of *S. coelicolor* A3(2). The cosmid is located between cosmids 2C4 and BAC8D1 (http://www.sanger.ac.uk/Projects/S_coelicolor/). DNA of cosmid 1G7, digested with *Dra*I and *Bam*HI, was screened with a probe having a sequence characteristic of acyltransferase active sites (Kuczek *et al.*, 1997). Under non-stringent conditions, six fragments were found to hybridize with the probe. These hybridizing fragments were cloned for sequence determination which, with the aid of CODONPREFERENCE analysis, revealed an ORF of 807 bp, located on two fragments and ending 265 bp from the T3 end of the 1G7 cosmid (Fig. 1a–c). The ORF, designated *scoT*, was deduced to encode a protein of 268 amino acid residues (molecular mass 28 686 Da; pI 6·17), of which about 53 % were predicted to be hydrophobic.

Comparison of the *scoT* sequence (GenBank AF109727) with others in the databases revealed extensive simi-

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                    *
scoT  81 DRPHAFFGHSMGALLAYELAREL - 113 aa - LRVLPGGHFYL
amy   85 DRPLALFGHSMGAIIGYELALRM - 113 aa - LRVLPGGHFFL
sac   81 DGPFALFGHSMGALIAYETARRL - 111 aa - TRTFPGGHFYL
sfr   85 GVPVALFGHSMGAVVAYETARLL - 113 aa - LRVFPGGHFYL
```

**Fig. 2.** Alignment of the amino acid sequence of the active-site region of the putative thioesterase II from *S. coelicolor* A3(2) ('scoT') with another TE: 'amy', TE II from the rifamycin biosynthetic gene cluster (AF040571) of *A. mediterranei*; 'sac', TE (X60379) from *Saccharopolyspora erythraea*; 'sfr', TE II (U08223) from the tylosin biosynthetic gene cluster of *S. fradiae*. Amino acids identical to those encoded by *scoT* are shown in bold. The conservative motif of the TE catalytic centre and the conservative histidine approx. 100 amino acids downstream are underlined; the active-site serine is indicated by an asterisk.

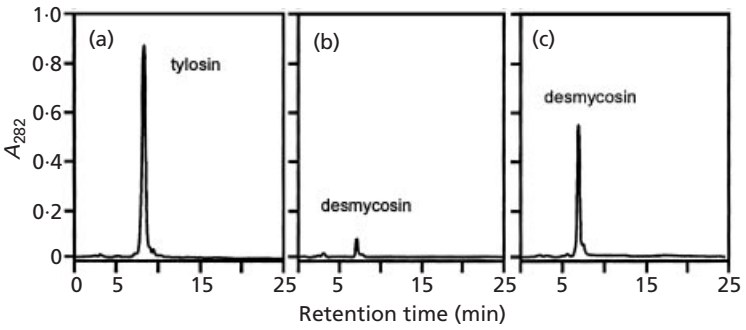


**Fig. 3.** HPLC analysis of fermentation products of *S. fradiae*. (a) Wild-type; (b) *tylO*-disrupted strain; (c) *tylO*-disrupted strain, complemented with *scoT*.

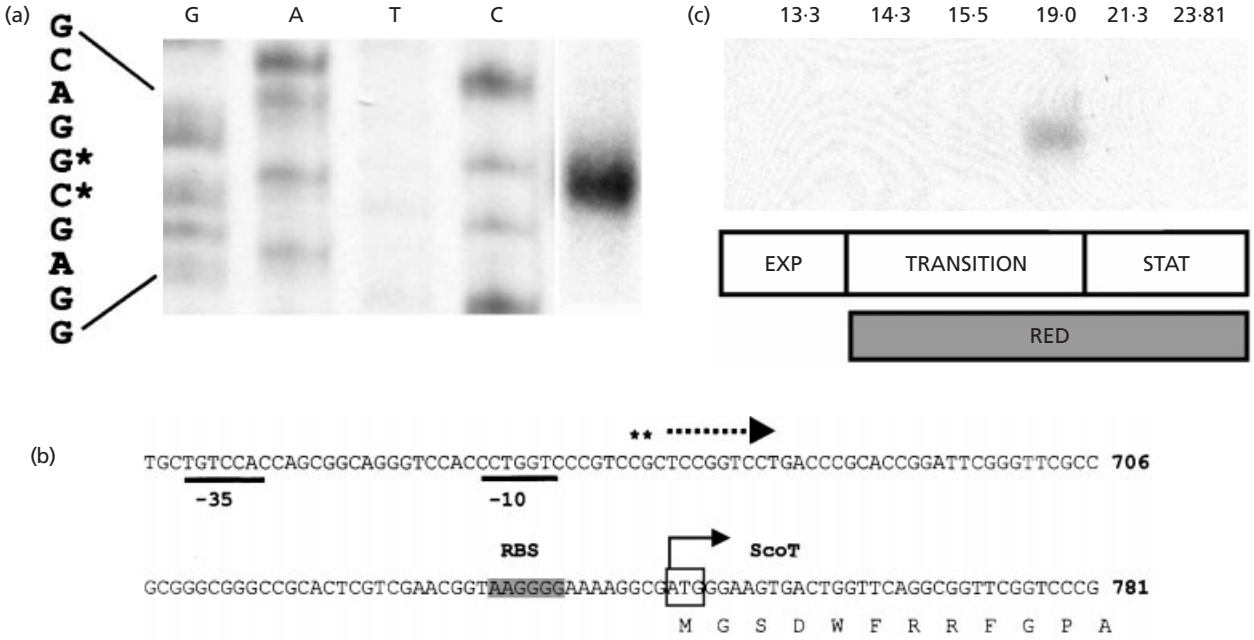


**Fig. 4.** $S_1$ nuclease transcript mapping of *scoT*. (a) $S_1$ nuclease mapping of the transcriptional start sites of *scoT*. A 430 bp PCR product (nt) labelled uniquely at the 5′ end position was used as a probe for *scoT* mRNAs. Asterisks indicate the probable transcription start points; the sequences shown are those of the template strand. Lanes T, G, C and A are sequence ladders derived from the same labelled primer that was used to generate the PCR product. (b) The promoter region of *scoT*. Possible −10- and −35-region sequences are underlined. A possible RBS is shown shaded. Transcriptional start points are indicated by asterisks, and the direction of transcription is indicated by an arrow. The translation start codon is shown boxed. The numbers are nucleotide positions as denoted in GenBank accession no. AF109727. (c) Analysis of the *scoT* transcript, using RNA isolated from a liquid SMM-grown culture of *S. coelicolor* A3(2) strain M145. 'EXP', 'TRANSITION' and 'STAT' indicate the exponential, transition and stationary phases of growth, respectively. The shaded box labelled 'RED' denotes the presence of undecylprodigiosin in the mycelium.

larities with TEs from various actinomycetes and other bacteria, and also with rat *S*-acyl fatty acid synthase complex. The greatest similarity was found with TE IIs (Pfam00975), i.e. 43% identity with a TE II (AF040570) from the rifamycin biosynthetic gene cluster of *Amycolatopsis mediterranei*, 43% identity with a TE II (X60379) associated with 6-deoxyerythronolide B synthase from *Saccharopolyspora erythraea*, and 40% identity with TylO (U08223), the TE II involved in tylosin biosynthesis in *Streptomyces fradiae*. ScoT is predicted to belong to the well-known alpha/beta hydrolase family (Pfam00975).

The ATG start codon of the *scoT* gene is preceded by a potential RBS sequence (AAGGGG) ending 8 bp before the start (nucleotides complementary to the 3′ end of the 16S rRNA from *Streptomyces lividans* are underlined) (Strohl, 1992) (see Fig. 4b). The ORF ends with a TGA stop codon. The amino acid motif GxSxG (x = any amino acid) characteristic of acyltransferases and TEs, with Ser-90 as the active-site residue, is present within the deduced sequence of the ScoT protein. A second conserved amino acid which might also be involved in catalysis is His-224 (Fig. 2). Usage of AGC as the codon for the active-site serine is typical of TE IIs [AGY for TE

II and TCN for TE I, where Y = (C, T), N = (A, C, G, T)] (Smith, 1994).

### Biological activity of TE II

We attempted to determine whether the product of *scoT* has TE II activity. This was examined by studying whether it could functionally replace the native TE II (i.e. TylO) in the tylosin producer, *S. fradiae*. ScoT and TylO show extensive amino acid sequence similarity, and the complementation system for the TE II gene-disrupted *S. fradiae* strain was constructed (Butler *et al.*, 1999). Therefore, *scoT* was cloned in a conjugative expression vector, pLST9828 (Fig. 1d), and integrated into the chromosome, at the *attB* site, of a *tylO*-disrupted strain of *S. fradiae* under the control of the strong, constitutive promoter *ermEp** (Butler *et al.*, 1999). This was done via conjugal transfer from *E. coli*, and three resultant transconjugant strains were subjected to fermentation analysis using quadruplicate cultures of each strain. Since TylO is co-expressed with the downstream gene, *tylCVI* (which encodes an enzyme involved in synthesis of the deoxyhexose sugar mycarose), disruption of *tylO* results in a polar effect on *tylCVI* expression, such that the *tylO*-disrupted strain produces demycarosyl-tylosin (desmycosin), as do strains in which the disruption is successfully complemented by cloned DNA (Butler *et al.*, 1999).

Thus, the *tylO* disruptant was complemented by *scoT*, as measured by the yield of desmycosin in fermentations analysed by HPLC with desmycosin as the internal standard (Fig. 3a–c). Desmycosin production was restored to up to 48 % of the level of macrolide produced by the wild-type strain (Fig. 3c). Because of the close similarity in the molar absorbance coefficients of tylosin and desmycosin, the amount of desmycosin produced as a fermentation product by a mutant strain was directly compared with the amount of tylosin produced by the wild-type strain. Control fermentation of the non-complemented, *tylO*-disrupted strain yielded only minimal amounts of desmycosin (Fig. 3b). These results showed that the TE II gene, *scoT*, from *S. coelicolor* A3(2) could, by complementation, restore macrolide production to a significant level in the *tylO*-disrupted strain of *S. fradiae*.

### $S_1$ nuclease protection experiments with *scoT* transcription

We examined if *scoT* undergoes transcription in the cells of its native host, *S. coelicolor* A3(2). Expression of *scoT* was analysed at different growth stages, using $S_1$ nuclease protection analysis of RNA extracted from *S. coelicolor* A3(2) M145 cultures grown in SMM liquid medium. Transcription start sites were identified 77–78 nt upstream of the translation start site of *scoT* (Fig. 4a). The apparent promoter −35 and −10 hexamer sequences are TGTCCA and CCTGGT, respectively, separated by a spacer region of 17 nt. The −10 hexamer sequence is separated from the +1 region by 6 nt. There is little similarity between the −35 and −10 regions of *scoT* and well-defined promoter regions with consensus sequences TTGAC(Pu) for the −35 region and TAG(Pu)(Pu)T for the −10 region (Strohl, 1992) (Fig. 4b).

Transcription of *scoT* was hardly detectable during exponential growth and early transition phase. The expression was induced at the 19th hour of growth, i.e. in late transition phase, and was quickly shut down in stationary phase (Fig. 4c).

## DISCUSSION

### ScoT is TE II protein

The translated sequence of *scoT* displayed extensive similarity to the deduced sequences of TE IIs associated with type I PKS complexes. Such similarity extended to several characteristics: (1) the overall amino acid sequence similarity; (2) the conservation of characteristic amino acid motifs; (3) the molecular mass of the peptide; (4) the distance from the amino terminus of the protein to the active-site serine (usually about 100 residues); (5) the position of the conserved histidine residue; (6) active-site serine codon AGC (Smith, 1994).

The amino acid sequences of all the known TE IIs are strongly conserved, especially in implicated catalytically active sites. The sequence data suggest that *scoT* encodes a TE II which, presumably, is catalytically active. However, in the absence of data relating to its substrate specificity, the probability that ScoT might be capable of functioning in association with a heterologous PKS system could not be predicted.

### ScoT complements the natural function of TE II in *S. fradiae*

ScoT was able to replace TylO, the TE II of the tylosin pathway, by restoring the efficiency of macrolide biosynthesis in the *tylO*-disrupted strain. The two enzymes appear, therefore, to be equivalent in their catalytic function. Interestingly, in their natural context, these enzymes are apparently associated with PKS enzymes that differ in module number and in the nature of the extender acyl units incorporated by the respective modules. The primary product of the tylosin PKS in *S. fradiae*, a linear polyketide derived from condensation of five propionate units, two acetate units and one butyrate unit (Baltz & Seno, 1988), undergoes cyclization into a 16-atom lactone ring, tylactone. This means that in tylactone biosynthesis, malonyl-CoA, methylmalonyl-CoA and ethylmalonyl-CoA are used as extender units. Although the polyketide product of the modular PKS with which ScoT putatively associates has not yet been determined, the sequences of the acyltransferase domains in each of the five extension modules of the PKS (Kuczek *et al.*, 1997; K. Pawlik, M. Kotowska & K. Kuczek, unpublished data) suggest that all five should use malonyl-CoA extender units according to the consensus sequence motifs correlated

with the substrate specificity of the acyltransferase domains (Haydock *et al*., 1995).

In any event, if ScoT is active as an editing enzyme with both the tylosin PKS and its natural PKS partner, it must be able to hydrolyse thioester bonds irrespective of the length of the extender units employed or the size of the nascent polyketide intermediates, since ScoT can apparently cooperate in the biosynthesis of a product longer than that of its native PKS partner.

We suggest that a mechanism other than substrate selectivity, probably based on differences in kinetic rates of hydrolysis by ScoT and polyketide condensation, is involved in competition between these two reactions. To clarify this, however, further studies which involve kinetic measurements of the enzyme activity are needed.

### Transcription of *scoT* occurs in a growth-phase-dependent manner

$S_1$ nuclease mapping of the *scoT* promoter region in *S. coelicolor* A3(2) showed that transcription was growth-phase dependent. It was detectable only during late transition phase, indicating the operation of a regulatory system that prevents expression of the gene throughout most of the growth cycle. Since production of secondary metabolites by *Streptomyces* is commonly initiated during transition phase, onset of *scoT* expression might be correlated with expression of the gene cluster encoding type I PKS, located close to *scoT*. Our results on this will be published in due course.

## ACKNOWLEDGEMENTS

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