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Characterization of type II thioesterases involved in natamycin biosynthesis in *Streptomyces chattanoogensis* L10

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ABSTRACT

The known functions of type II thioesterases (TEIIs) in type I polyketide synthases (PKSs) include selecting of starter acyl units, removal of aberrant extender acyl units, releasing of final products, and dehydration of polyketide intermediates. In this study, we characterized two TEIIs (ScnI and PKSIaTEII) from *Streptomyces chattanoogensis* L10. Deletion of *scnI* in *S. chattanoogensis* L10 decreased the natamycin production by about 43%. Both ScnI and PKSIaTEII could remove acyl units from the acyl carrier proteins (ACPs) involved in the natamycin biosynthesis. Our results show that the TEII could play important roles in both the initiation step and the elongation steps of a polyketide biosynthesis; the intracellular TEIIs involved in different biosynthetic pathways could complement each other.

1. Introduction

Many medicines and biologically active agents are biosynthesized by polyketide synthases (PKSs). PKSs can be classified into three types based on their domain organizations. A type I PKS is organized into modules, each of which harbors three core domains, a β-ketoacyl synthase (KS), an acyltransferase (AT), and an acyl carrier protein (ACP), as well as some optional domains including a ketoreductase (KR), a dehydratase (DH), an enoylreductase (ER), etc [1–3]. A type II PKS contains several single-domain proteins, minimally a KSα, a KSβ (also named as chain-length factor), and an ACP [4]. A type III PKSs contains only a KS, which acts on the acyl-CoAs directly, independent of ACP [5]. Typical polyketide biosynthetic steps catalyzed by a type I PKS include: (1) an AT transfers a (2-substituted)malonyl unit into a holo-ACP via an acyl-*O*-AT intermediate, resulting in a (2-substituted)malonyl-*S*-ACP; (2) a KS condenses the upstream acyl unit and the carbanionic acyl-*S*-ACP resulting from decarboxylation of the (2-substituted)malonyl-*S*-ACP, forming a β-ketoacyl-*S*-ACP and extending the carbon skeleton of polyketide intermediate with two carbons; (3) a KR, a DH, and an ER in turn catalyze reducing the β-ketoacyl-*S*-ACP to form a β-hydroxyacyl-*S*-ACP, dehydrating the β-hydroxyacyl-*S*-ACP to form a α,β-enoyl-*S*-ACP, reducing the α,β-enoyl-*S*-ACP to form a saturated acyl-*S*-ACP, respectively; (4) a thioesterase (TE) releases the final product by hydrolysis or cyclization of the intermediate that is covalently linked to the ACP in the termination module.

In bacteria, the related genes of a secondary metabolite usually locate together as a gene cluster. A type I PKS gene cluster usually includes one type I thioesterase (TEI) gene and one type II thioesterase (TEII) gene. Unlike TEIs that are integrated at the C terminus of termination modules, TEIIs are discrete proteins. The known function of TEIs is to release final products [1,3,6]; however, the functions of TEIIs in type I PKSs are various. Some TEIIs can play selecting roles of the starter acyl units in the initiation biosynthetic steps. Ery-ORF5 and ScoT show much higher activity to remove the unfavored starter acyl units than the favored starter acyl units from the ACPs in the initiation modules [7–10]. Most TEIIs play editing roles by removal of the aberrant extender acyl units in the elongation biosynthetic steps. RifR, FscTE, and TylO show much higher activity to hydrolyze the aberrant acyl units, which probably come from premature decarboxylation of the extender units or from mispriming of ACP domains, than the correct ones from the ACPs in the elongation modules [11–14]. Interestingly, few TEIIs provide the activities of missing TEI *in trans*, which play essential roles in releasing of the final products from the ACPs in the termination modules in the last biosynthetic steps.

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Nanchangmycin biosynthetic PKS and enediyne biosynthetic PKS lack TEIs in the termination modules. Instead, releases of the final products from these two PKSs are catalyzed by TEIIs [15–16]. Recently, Fr9-TE2, a TEII involved in the FR901464 biosynthesis, has also been reported to catalyze dehydration of the polyketide intermediate to yield a *cis*-double bond [17]. The functions of TEIIs in type II PKSs and non-ribosomal peptide synthetases (NRPSs) have also been characterized [18–22].

Natamycin (NTM) (also named as pimaricin), a widely used antifungal agent in both human therapy and the food industry, is a polyene macrolide biosynthesized by the type I PKSs from several *Streptomyces* strains, such as *Streptomyces natalensis*, *Streptomyces gilvosporeus*, and an industrial producer, *Streptomyces chattanoogensis* L10 [23–28]. The NTM biosynthetic PKS in *S. chattanoogensis* L10 (*scn* PKS) contains a terminal TEI. In this study, we identified and characterized two discrete TEII genes (*scnI* and *PKSIaTEII*) within two type I PKS gene clusters from *S. chattanoogensis* L10. Our results here show that these TEIIs may play important roles in both the initiation step and the elongation steps of a polyketide biosynthesis; the intracellular TEIIs involved in different biosynthetic pathways could complement each other.

2. Materials and methods

2.1. Bacterial strains, plasmids, culture conditions, and general techniques

Bacterial strains and plasmids used in this study are listed in Table 1. Primers used in this study are listed in Table S1. The spore preparation of *S. chattanoogensis* L10 on YMG agar, DNA manipulations in *S. chattanoogensis* L10, *Escherichia coli-Streptomyces* conjugation, fermentation of *S. chattanoogensis* L10 and its recombinant strains in YEME with 4% glucose, and the quantification of NTM production were performed as described previously [23–26].

2.2. In vitro hydrolysis of acyl-S-ACPs catalyzed by TEIIs

A typical acyl-*S*-ACP biochemical synthetic reaction mixture of 50 μl, containing 16 μM apo-ACPs (*scn* ACP0-1, *scn* ACP0-2, *scn* ACP7, or *scn* ACP10), 160 μM acyl-CoA (acetyl-CoA, propionyl-CoA, malonyl-CoA, or methylmalonyl-CoA), 1.6 μM Sfp [29], 1.25 mM $MgCl_2$, and 100 mM Tris–HCl (pH 8.0), was incubated at 25 °C for 30 min. To the above reaction mixture, TEII (ScnI, PKSIaTEII, $PKSIaTEII_{S69A}$, $PKSIaTEII_{R174A}$, or $PKSIaTEII_{R174H}$) was added to the final concentration of 1.6 μM and then incubated at 25 °C for 1 h.

2.3. Construction of mutants in S. chattanoogensis L10

The *scnI* was deleted from *S. chattanoogensis* L10 by using a λ-RED-mediated PCR targeting system, resulting in strain sHJ001 [30]. The *PKSIaTEII* was deleted from sHJ001, resulting in strain sHJ015. The experimental details are described in the Supplementary information.

The *scnI* gene was cloned as an *Nde*I/*Not*I fragment from pHJ0019 into the same sites of pIJ8660 [31], a site-specific integration vector, containing *ermEp** promoter, φ31 *int* and *attp*, resulting in the plasmid pYY0030. The pYY0030 was introduced into *E. coli* ET12567 (pUZ8002) and then was transferred into *S. chattanoogensis* L10 via conjugal transfer by standard procedures. The resulting mutant sHJ014 was screened with apramycin resistance and confirmed by PCR analysis using primers Pri53/scnI3.

3. Results

3.1. The important role of ScnI in natamycin production

The *scn* PKS gene cluster from *S. chattanoogensis* L10 involved in the biosynthesis of NTM contains one TEII gene *scnI* in the middle of the gene cluster [25]. Protein sequence alignment of ScnI with several known TEIIs shows that ScnI contains a conserved GXSXG motif and a characteristic Ser-His-Asp catalytic triad (Fig. 1) [7,15]. To determine the role of ScnI in NTM production in vivo, *scnI* was in-frame deleted in *S. chattanoogensis* L10 by using Redirect technology [30], resulting in strain sHJ001 (Fig. S1). The sHJ001 was fermented in YEME liquid medium with 4% glucose in flasks in triplicate by using *S. chattanoogensis* L10 as a control as described previously [23–26]. HPLC analysis showed the NTM yield of sHJ001 was around 57% of that of *S. chattanoogensis* L10, confirming that ScnI plays important roles in NTM production (Fig. 2).

The *scnI* gene overexpression strain sHJ0014, in which an extra *scnI* was under the control of the *ermEp** promoter in *S. chattanoogensis* L10, was also constructed and confirmed by PCR (Fig. S1). Fermentation of sHJ014 in triplicate showed the NTM yields of sHJ014 increased about 22% compared to that of *S. chattanoogensis* L10 (Fig. 2).

3.2. In vitro characterization of ScnI

To determine the function of ScnI in vitro, ScnI was overproduced in *E. coli* and purified to homogeneity (Fig. S2). Four apo-ACPs from *scn* PKS were prepared for productions of acyl-ACPs (the substrates of ScnI), including *scn* ACP0-1 (the first ACP domain in the initiation module for loading of acetyl unit), *scn* ACP0-2 (the second ACP domain in the initiation module for loading of acetyl unit), *scn* ACP7 (the ACP domain in the seventh elongation module for loading of methylmalonyl unit), and *scn* ACP10 (the ACP domain in the tenth elongation module for loading of malonyl unit) [23,32–33]. Twelve acyl-*S*-ACPs were biochemically synthesized by incubation of apo-ACPs with acyl-CoAs (acetyl-CoA, propionyl-CoA, malonyl-CoA, and methylmalonyl-CoA) and a known

Table 1
The strains and plasmids used in this study.

	Description	References
Strains		
S. chattanoogensis L10	Industrial natamycin producer	[27,30–32]
sHJ001	In-frame deletion of *scnI* in *S. chattanoogensis* L10	This study
sHJ014	Overexpression of *scnI* in *S. chattanoogensis* L10	This study
sHJ015	In-frame deletion of *scnI/PKSIaTEII* in *S. chattanoogensis* L10	This study
Plasmids		
37A8	Cosmid pHAQ31 carrying the *scn* PKS gene clusters	This study
pHJ0019	*ScnI* cloned as an *Nde*I/*Hin*dIII fragment into pET28a	This study
pYY0030	*ScnI* cloned as an *Nde*I/*Not*I fragment into pIJ8660	This study
pYY0064	*PKSIaTEII* cloned as an *Nde*I/*Hin*dIII fragment into pET28a	This study
pYY0065	$PKSIaTEII_{S69A}$ cloned as an *Nde*I/*Hin*dIII fragment into pET28a	This study
pYY0066	$PKSIaTEII_{R174A}$ cloned as an *Nde*I/*Hin*dIII fragment into pET28a	This study
pYY0067	$PKSIaTEII_{R174H}$ cloned as an *Nde*I/*Hin*dIII fragment into pET28a	This study