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Identification of NanE as the Thioesterase for Polyether Chain Release in Nanchangmycin Biosynthesis

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Summary

The polyketide synthase (PKS) for the biosynthesis of the polyether nanchangmycin lacks an apparent thioesterase comparable to the type I thioesterase domains of the modular PKSs responsible for macrolide biosynthesis. Three candidate polyether chain-releasing factors were examined. Both the putative CR domain and the NanE protein appeared to be genetically relevant. Among the three heterologously expressed soluble proteins (recombinant CR domain, the ACP-CR didomain, and NanE) tested, only NanE hydrolyzed the polyether-SNAC. By contrast, recombinant DEBS TE from the erythromycin pathway, and the recombinant MonAX, a type II TE associated with the polyether monensin biosynthesis for which a homolog has not been detected in the nanchangmycin cluster, hydrolyzed a diketide-SNAC but not the polyether-SNAC. We could thus conclude that NanE is a dedicated thioesterase mediating the specific release of the polyether chain during nanchangmycin biosynthesis.

Introduction

Polyether ionophores have found widespread application in veterinary medicine and animal husbandry and more recently have been identified as agents with activity against drug resistant strains of malaria [1, 2]. Hundreds of polyethers, such as monensin, salinomycin, nigericin, and nanchangmycin [3, 4], have the ability to selectively chelate metal ions and transport them across cell membranes [5]. Nanchangmycin (also known as dianemycin), produced by *Streptomyces nanchangensis*, has been found to inhibit gram-positive bacteria and can be used as a growth promotant in poultry and to cure coccidiosis in chickens [6]. Because of its strong activity and low toxicity, nanchangmycin has become a potential drug for the poultry industry.

Two gene clusters for the biosynthesis of polyether antibiotics have been identified and sequenced, one of which encodes the biosynthesis of nanchangmycin [7] and the other of monensin [8]. The deduced genetic organization indicated that the polyketide skeletons of nanchangmycin and monensin were assembled by large modular polyketide synthases, analogous to the well-characterized type I PKSs of macrolide biosynthesis. Interestingly, neither of the two modular polyether PKSs appears to include a type I thioesterase domain of the type usually located at the C terminus of the last polyketide chain-extension module. The conversion of the parent polyketide to the corresponding polyether appears to involve an oxidative cyclization [7, 8], in which epoxidation of the polyketide double bonds is followed by a cascade of stereospecific ring closures [9]. Recently, Leadlay's group has demonstrated that the parent premonensin triene has the *E,E,E* geometry [10, 11], consistent with the polyether cyclization mechanism originally proposed by Cane and Westley [12]. A unique cassette of genes is found only in the biosynthetic gene clusters of the polyethers nanchangmycin (*nanO*, *nanI*, and *nanE*) and monensin (*monCI*, *monBI/monBII*, and *monCII*, respectively). MonCI, the homolog of NanO, has been shown to be a flavin-dependent epoxidase that is required to convert the all-*E* premonensin triene to the corresponding triepoxide [8, 11]. MonBI and MonBII, the homologs of NanI, have been proven to be the key epoxide hydrolase/cyclases that are responsible for formation of the monensin polyether rings [13].

Surprisingly, neither the nanchangmycin nor monensin PKSs contains an obvious intrinsic thioesterase (TE) domain that normally would be located at the C terminus of the final PKS extension module. Three candidates for the requisite thioesterase activity can be considered [7, 8] (Figure 1). (1) The CR domain (for "chain-releasing") is fused downstream of ACP14 in the final module of the nanchangmycin PKS. The amino acid sequence of the CR domain shows homology with a large superfamily of metalloenzymes, with the highest sequence identity (28.8%) being with TatD [14], a DNase with esterase activity. The location of this domain and its predicted biochemical function suggested that this domain might be responsible for hydrolytic chain release of the mature polyether product from the PKS [7, 8]. (2) The hypothetical NanE protein also merits consideration as the potential polyether thioesterase. Although NanE does not show significant sequence similarity to known enzymes, NanE contains a serine protease-type hydrolytic motif including a Ser-Asp-His catalytic triad, also found in type I thioesterases of the α/β hydrolase family such as those found in PKS TE domains. An analogous module-independent hydrolase, Ayg1p, with a similar catalytic triad has been purified from *Aspergillus oryzae* and shown to be responsible for product release in the hydrolytic conversion of the heptaketide naphthopyrone YWA1 to the pentaketide T4HN and acetoacetic acid [15, 16]. (3) Two type II TE genes (*monAIX* and *monAX*) have been identified at either end of the *mon* cluster [8]. Inactivation of type II TEs in other PKS gene clusters has been shown to decrease antibiotic production significantly [17–19], and in several cases, such type II TEs had been shown to act as editing enzymes that remove incorrect acyl thioesters from the ACP, both in vivo and in vitro [20–22]. In principle, similar type II

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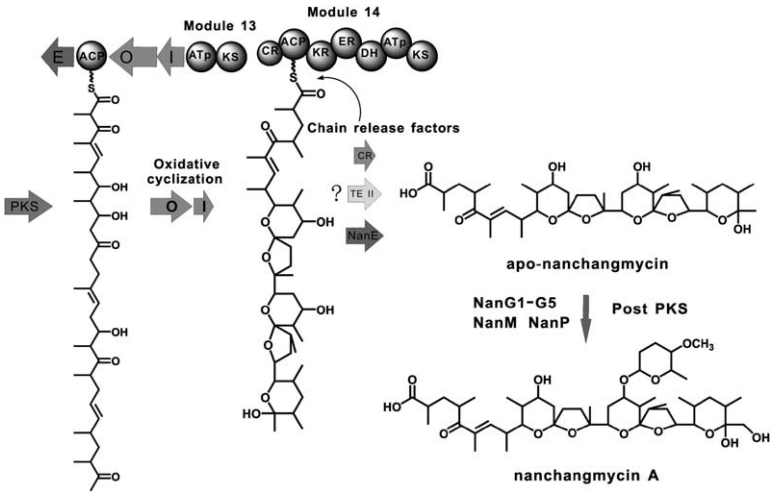

Figure 1. Proposed Final Steps of Nanchangmycin Biosynthesis

Three suspected factors (CR, TE II, and NanE) responsible for releasing the polyether chain from NANS are indicated with a question mark (?).

TEs in the monensin gene cluster might be responsible for release of the polyether chain in monensin biosynthesis. Although no such hypothetical type II TE has been found in the corresponding *nan* gene cluster, it could not be excluded a priori that the hypothetical TEII function might be recruited from elsewhere in the genome.

We have used a combination of genetic and biochemical approaches to investigate the properties of each of the above putative polyether chain-releasing thioesterases. Our results establish conclusively that NanE is responsible for polyether chain release in nanchangmycin biosynthesis and rule out the involvement of either the CR or type II TE domains in this process.

Results

The CR Domain Is Required for Efficient Nanchangmycin Production In Vivo, a Function that Can Not Be Replaced by Type I TEs

The polyether *apo*-nanchangmycin, which lacks the glycosyl substituent, might be released from the ACP of module 14 of the nanchangmycin PKS prior to modification by the glycosyltransferase NanG5 [7] (Figure 1). In module 14, the ACP domain is immediately followed by a hypothetical CR domain, whose location is analogous to that of type I TE domains in typical macrolide PKSs. In fact, targeted in-frame deletion of the 61 amino acids of the hypothetical CR domain gave a strain, *S. nanchangensis* SL1 (Figure 2A), that retained the ability to produce nanchangmycin A, albeit at a ca. 1000-fold decreased level compared with the wild-type strain NS3226, as measured by LC/ESI-MS analysis (Figure 3).

To test whether the decrease in the yield of nanchangmycin A in SL1 was actually caused by the in vivo deletion of the CR domain, a 1429 bp BamHI fragment from cosmid 19B4, covering a complete final PKS gene including the natural CR domain, was cloned into pHZ1358 (creating pJTU1339) before introduction by conjugation into SL1. The level of nanchangmycin production in the resulting exconjugant was restored to near wild-type levels (data not shown). The *in trans* complementation of the CR deletion, with restoration of nanchangmycin production, suggested that it was indeed the deletion of the CR domain, and not an unknown chromosomal mutation, that had suppressed the antibiotic production level.

We also attempted to complement the CR deletion by introducing a CR domain under the control of a constitutively expressed *ermE** promoter. To this end, the CR domain was inserted into the NdeI and XbaI sites of pIB139 (to create pJTU1340) before conjugation into strain SL1 by site-specific integration into the *attB* site of the SL1 chromosome mediated by the ϕC31 *attP* site of pJTU1340. The level of nanchangmycin production in the resultant recombinant strain (LTG6) was actually lower than that of the ΔCR mutant strain SL1.

Since earlier work on erythromycin polyketide biosynthesis had established that recombinant DEBS ACP6-TE didomain had similar hydrolytic ability to the DEBS TE domain alone [23], we also tested whether the corresponding ACP14-CR didomain could complement the CR deletion. Plasmid pJTU1341 carrying the ACP14-CR didomain under control of the constitutive *ermE** promoter was introduced into strain SL1. The resultant SL1::pJTU1341 with the ACP14-CR didomain expressed *in trans* failed to restore nanchangmycin production to normal levels. The observed decrease in antibiotic production was apparently not due to the in *trans* effect of the CR domain, however, since control experiments revealed that simple plasmid vector integration mediated by the ϕC31 *attP* site also decreased antibiotic production level in strains NS3226::pIB139 and SL1::pIB139.

To test whether the CR domain could be replaced by a type I PKS TE, we chose to fuse the DEBS TE from the erythromycin PKS and the AVE TE from the avermectin PKS downstream of ACP14 (to generate mutant LTG18) or inside the XhoI site of the CR domain (to generate mutant SYH30), respectively. Neither of these two mutants, whose phenotype was the same as the in-frame ΔCR mutant described above, restored nanchangmycin productivity.

NanE Is a Required Element for the Biosynthesis of Nanchangmycin

To assess the role of *nanE* in nanchangmycin biosynthesis, a 558 bp internal DNA fragment, coding for 186 aa, was deleted in frame so as to remove the entire catalytic triad presumed to be essential for thioester hydrolysis

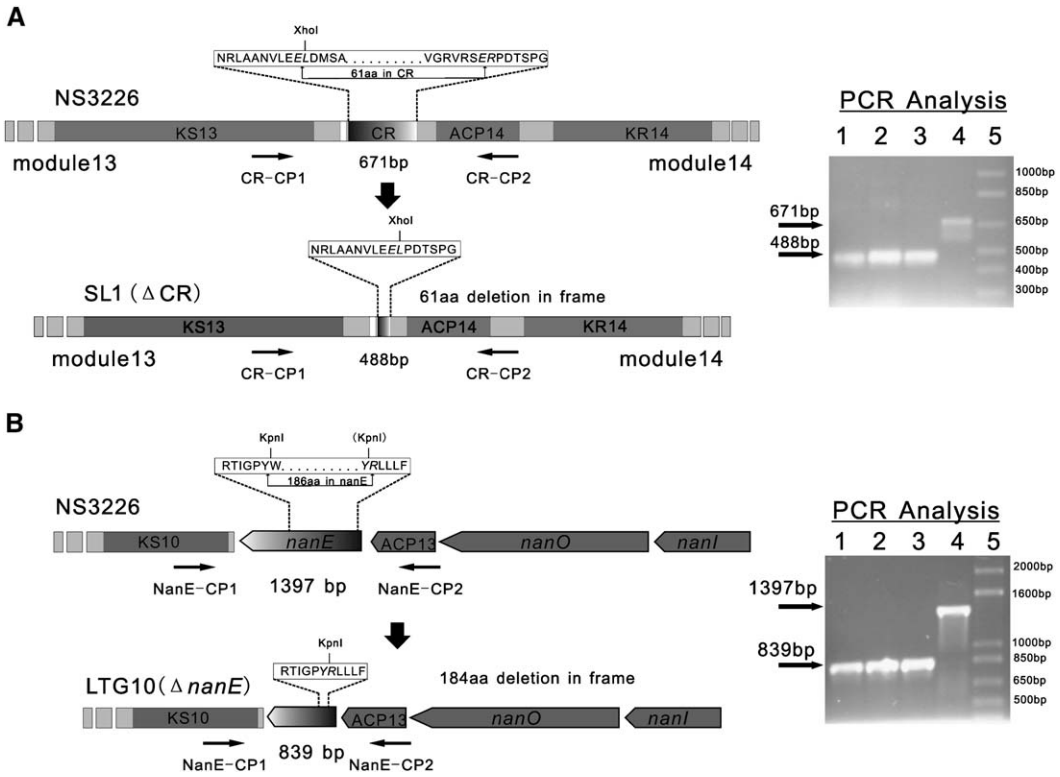

Figure 2. In-Frame Deletion of 61 aa from the CR Domain to Generate the ΔCR Mutant SL1 and a 186 aa Deletion in NanE to Generate a *ΔnanE* Mutant LTG10 in *S. nanchangensis* NS3226

The desired mutations were confirmed by PCR analysis (shown at right side of each mutation). Lanes 1, 2, and 3 show PCR with three individuals for each mutant (SL1-3 for SL1, and LTG10-1-3 for LTG10) as templates, respectively. Lane 4 is the PCR analysis with wild-type NS3226 as the control, and lane 5 is the DNA size standard.

(Figure 2B). To this end, the conjugation plasmid pJTU1364, carrying a fragment with the 558 bp deletion, was introduced into wild-type *S. nanchangensis* NS3226. After initial selection for Thio^R exconjugants followed by two rounds of growth in the absence of thiostrepton, 3 Thio^s colonies (LTG10-a, -b, and -c) out of 58 were selected by a PCR screen for double crossover events and confirmed as Δ*nanE* mutants. Nanchangmycin A could not be detected by HPLC analysis of the methanol extracts obtained from surface cultures of the three independent Δ*nanE* mutant strains, although trace amounts could still be detected by LC/ESI-MS (Figure 3).

The requirement for *nanE* in the biosynthesis of nanchangmycin was further supported by the fact that Δ*nanE* mutant could be complemented *in trans*. This was achieved by ligation of the PCR-amplified *nanE* gene into the NdeI and XbaI sites of pIB139 so that *nanE* expression was under the direct control of the constitutive *ermE** promoter in plasmid vector pJTU1366, which was subsequently introduced into a Δ*nanE* mutant LTG-10a by conjugation. LC-MS analysis of the fermentation extracts from the resulting *S. nanchangensis* LTG11 carrying pJTU1366 clearly demonstrated that nanchangmycin production had been restored to a level ca. 80% that of the wild-type strain NS3226 (Figure 3).

Heterologous Expression of the CR and NanE Proteins in *E. coli*

In order to examine the potential role of the CR and NanE proteins in releasing the polyether from the ACP of the nanchangmycin PKS, the individual recombinant proteins were expressed and assayed in vitro for thioesterase activity. The requisite constructs for expression of recombinant CR domain and NanE protein (pJTU1355 and pJTU1373, respectively) were prepared by incorporating the appropriate PCR-amplified 343 and 873 bp fragments into the corresponding sites of pET15b and pET28a. The resulting soluble His_6-tag fusion proteins were isolated from *E. coli* BL21 (DE3)/pLysE. We also expressed the ACP14-CR didomain as well as the type II TE obtained by amplification of *monAX* from the genome of *S. cinnamonensis*. For comparison, we also expressed the previously described DEBS TE from the erythromycin PKS with a pET28a-derivative in *E. coli* BL21(DE3)/pLysE [24].

The M_r of NanE (34,000), DEBS TE (32,000), and MonAX (32,000 Da), as determined by SDS-PAGE, agreed well with the predicted molecular masses of 34,018.32 Da for NanE, 32,489.23 Da for DEBS TE, and 32,084.04 Da for MonAX (Figure 4C). Gel permeation chromatography on a Superdex 75 column of the three purified proteins indicated that they all were homodimers (data not shown). The apparent M_r values of both the recombinant CR (ca. 18,000) and ACP-CR (ca. 29,000) domains, however, were considerably larger than their predicted molecular masses (CR, 13,347.0 Da; ACP-CR, 21,575.9 Da) (Figures 4A and 4B). When the two CR-containing proteins were preincubated overnight in storage buffer containing 2 mM EDTA, the observed molecular sizes were consistent with the

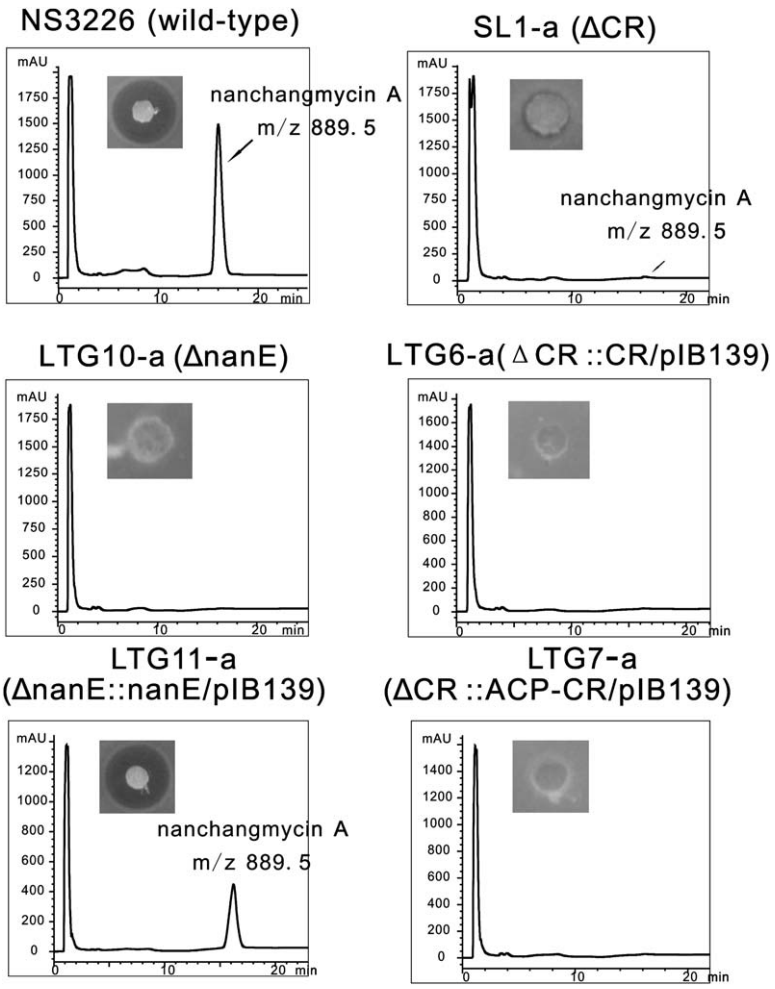

Figure 3. Detection of Nanchangmycin Productivity Using Different Mutants by Bio-Assay Using *Bacillus cereus* 1126 as an Indicator Strain and by LC/MS

The peak corresponding to nanchangmycin A (R_t = 17 min) was identified by its *m/z* [M+Na$^+$] = 889.5, whose presence correlated with the observed inhibition zone.

predicted masses. Analysis by MALDI-TOF-MS of the purified CR domain carrying an N-terminal His_6-tag revealed a major species with a molecular mass of 13,281.63 Da, in reasonable agreement with the predicted molecular mass 13,347.0 Da for recombinant protein lacking the N-terminal methionine and carrying a single Zn^{2+} ion.

In Vitro Enzymatic Assay of Recombinant CR and NanE Proteins Using Diketide and Polyether-SNACs as Substrates

Thioesterase-catalyzed polyether release, which is assumed to be mechanistically very similar to the release of type I PKS products catalyzed by macrolide TE domains, is thought to involve two discrete enzymatic steps: transfer of the acyl chain from the thiol of the phosphopantetheine arm of the ACP of the PKS to the serine hydroxyl group in the TE active site, followed by hydrolytic cleavage of the intermediate acyl-*O*-serine ester [25]. The *N*-acetylcysteamine (SNAC) thioester derivative of nanchangmycin, prepared from nanchangmycin A, served as analog of the natural ACP bound substrate. The (*2S*,*3R*)-2-methyl-3-hydroxypentanoyl-SNAC thioester (diketide-SNAC), which is hydrolyzed efficiently in vitro by both the DEBS TE and PICS TE [26], was used as a comparison substrate. Incubations with nanchangmycin-SNAC were carried out in the presence of 5% methanol, due to poor solubility in water of the hydrophobic polyether thioester. Addition of NanE resulted in efficient hydrolysis of nanchangmycin-SNAC (Figure 5B), but little detectable hydrolysis of the short chain diketide-SNAC (Figure 5A). Neither nanchangmycin-SNAC

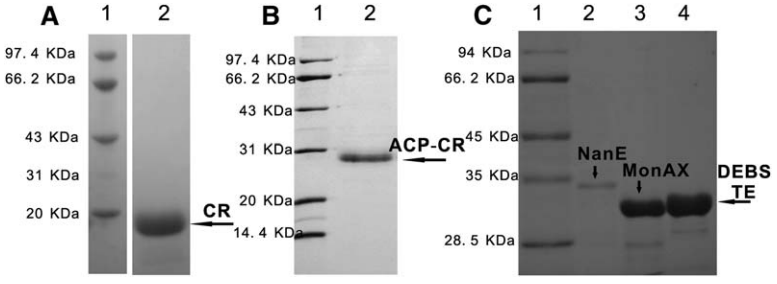

Figure 4. SDS-PAGE Analysis of the Purified Proteins Carrying CR, ACP-CR, NanE from *S. nanchangensis* NS3226, MonAX from *S. cinnamonensis*, and DEBS TE from *S. erythraeas*

Lane 1 in each column is the respective protein size standards. The detected sizes for proteins carrying CR (ca. 18 kDa) and ACP-CR (ca. 28 kDa) reverted to their predicted sizes (CR = 13,347.0 Da, and ACP-CR = 21,575.9 Da) after storage overnight in buffer containing 2 mM EDTA (not shown).

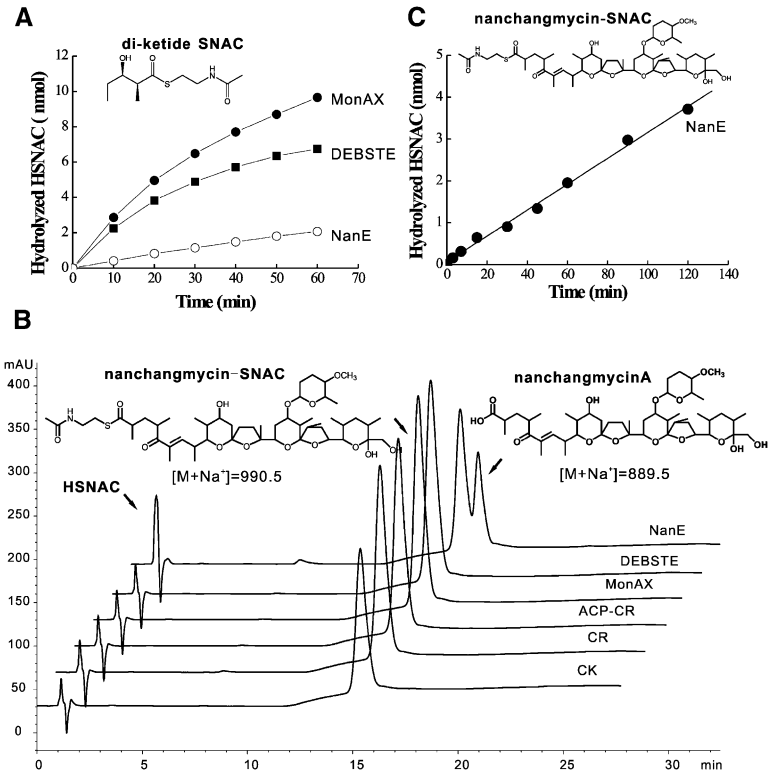

Figure 5. Detection of Hydrolytic Activity of NanE toward SNAC Esters

(A) Hydrolysis of diketide-SNAC (top of [A]) could be detected with MonAX and DEBS TE in the presence of DTNB, but the rate is very low with NanE (5 mM substrate).

(B) Hydrolysis of nanchangmycin-SNAC (top left of [B]) into nanchangmycin (top right of [B]) could be detected by HPLC/MS for NanE, but not with DEBS TE, MonAX, ACP-CR, or CR.

(C) Kinetic analysis of in vitro hydrolysis of nanchangmycin-SNAC (38.4 μM) by His_6-NanE. The amount of nanchangmycin (solid circle) produced at each time point was quantitated by RP-HPLC. The initial velocity (V_0) was calculated to be 3.1×10^{-2} nmol/min.

nor diketide-SNAC was hydrolyzed by the recombinant CR domain or ACP-CR didomain, with or without added metal ions (Figure 5B). By contrast, both DEBS TE and MonAX could hydrolyze the diketide-SNAC but not nanchangmycin-SNAC, with the hydrolytic activity of the MonAX toward diketide greater than that of DEBS TE (Figure 5A) [24]. The ability to hydrolyze a branched chain diketide SNAC indicates that MonAX is not a typical PKS type II TE since such enzymes normally show preferential hydrolysis of short, straight-chain acetyl- or propionyl-thioesters that can result from aberrant decarboxylation of malonyl- or methylmalonyl-ACP [20, 21].

Due to poor substrate solubility, even in the presence of added Tween 80 and methanol, the rate of the His6-NanE-catalyzed hydrolysis of nanchangmycin-SNAC was determined with a low substrate concentration of 38.4 μM in the presence of 5% methanol in 500 μl phosphate buffer (pH 8.0). Under these conditions, the initial velocity V_0 of the hydrolysis of for nanchangmycin-SNAC was 3.1×10^{-2} nmol/min (Figure 5C). Under the same conditions, hydrolysis of diketide-SNAC could not be detected.

Discussion

The above results demonstrate conclusively that NanE, encoded by a single ORF located between the genes for modules 10 and 14 of the nanchangmycin PKS, is the thioesterase that is responsible for the hydrolytic release of the nanchangmycin polyether chain. The results also rule out the possibility that the polyether chain-releasing thioesterase is either the CR domain, which is located at the C terminus of the last module of the nanchangmycin PKS [7], or an unidentified type II TE protein [8].

Thioesterases (TEs) responsible for polyketide chain termination and release have been well characterized from PKS, NRPS, and PKS/NRPS systems [27–29]. All of the latter thioesterases have very low amino acid sequence similarity to NanE. Nonetheless, NanE and the type I TE domains of modular PKSs and NRPSs do appear to belong to a common α/β hydrolase superfamily. The evolutionary distance between NanE and other α/β hydrolases, as revealed by the phylogenetic tree in Figure 6, clearly suggests that the type I PKS TEs, type I NRPS TEs, type II PKS TEs, and type II NRPS TEs are each clustered within their respective family groups, while NanE is distantly related to all of them. By contrast, NanE is closely related to both MonCII [8], the thioesterase recently identified in the monensin biosynthetic pathway, and the homologous NigCII, which plays an analogous role in nigericin bioynthesis (NCBI accession number DQ354110), suggesting that the polyether thioesterases form a distinct subfamily.

Members of the TE family of α/β hydrolases contain a characteristic Ser, His, Asp catalytic triad, exemplified by the Ser142, His259, and Asp169 that line the active-site channel of the DEBS TE [30]. According to the accepted mechanism for DEBS TE-catalyzed product release, His259, stabilized by Asp169, acts as a catalytic base to remove a proton from Ser142, which attacks the ACP bound polyketide acylthioester substrate to yield a transient covalent acyl-*O*-serine intermediate. The acyl enzyme then undergoes intramolecular nucleophilic attack by a substrate hydroxyl group to generate the macrolide lactone aglycone. Alignment of members of the polyether family of thioesterases, NanE, MonCII, and NigCII, with the TE domains of DEBS and AVE as well as with MonAX shows that the polyether TEs all possess an active site Ser (Ser96 in NanE) as part of

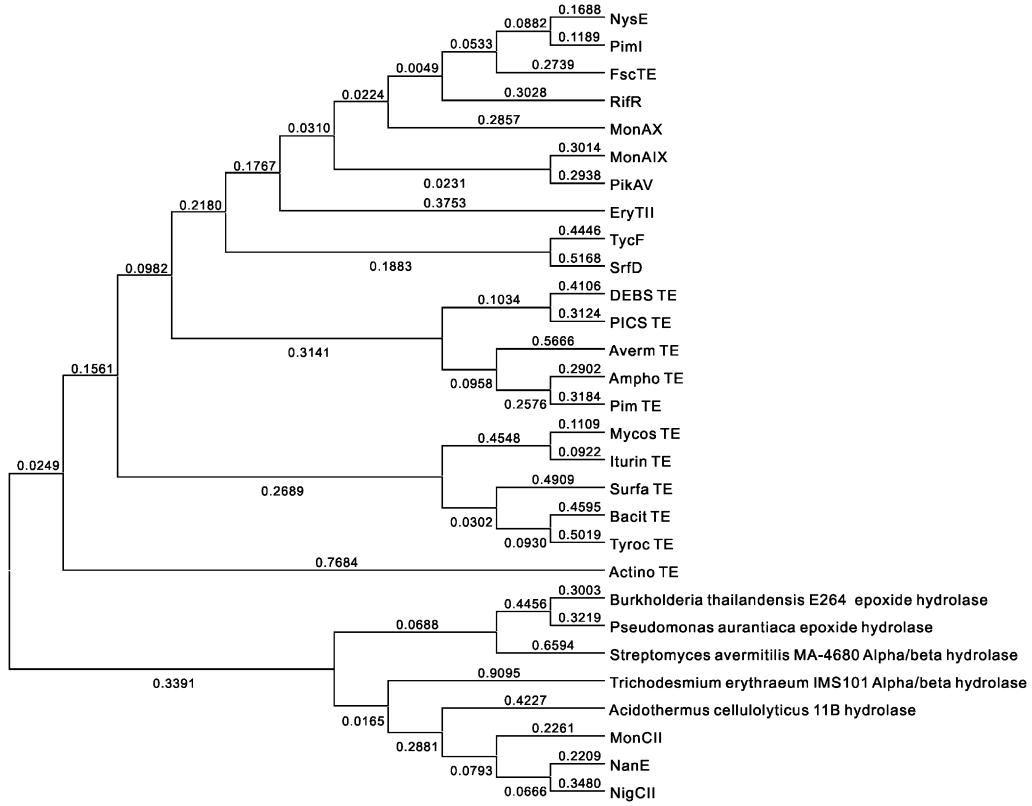

Figure 6. Phylogenetic Tree Geneterated Using MEGA 3.1 Representing the Apparent Evolutionary Distances of Thioesterases and Other α/β Hydrolases

NysE, a type II TE in nystatin cluster, AF263912; PimI, a type II TE in pimaricin cluster, AJ278573; FscTE, a type II TE in candicidin cluster, AY310323; RifR, a type II TE in rifamycin cluster, AF040570; MonAX and MonAIX, two type II TE in monensin cluster, AF440781; PikAV, a type II TE in methymycin/pikromycin cluster, AF079138; EryTII, a type II TE in erythromycin cluster, AY623658; TycF, a type II TE in tyrocidine NRPS cluster, AF004835; SrfD, a type II TE in surfactin NRPS cluster, AF233756; DEBS TE, a type I TE in erythromycin cluster, X62569; PICS TE, a type I TE in methymycin/pikromycin cluster, AF079138; Averm TE, a type I TE in avermectin cluster, NC_003155; Ampho TE, a type I TE in amphotericin B cluster, AF357202; PimTE, a type I TE in pimaricin cluster, AJ278573; Mycos TE, a type I TE in a NRPS/PKS hybrid mycosubtilin cluster, AF184956; Iturin TE, a type I TE in NRPS/PKS hybrid Iturin cluster, AB050629; Surfa TE, a type I TE in NRPS surfactin cluster, AF233756; Bacit TE, a type I TE in NRPS bacitracin cluster, AB016966; Tyroc TE, a type I TE in NRPS tyrocidine cluster, AF004835; Actino TE, a type I TE in NRPS actinomycin cluster, AF047717; *Burkholderia thailandensis* E264 epoxide hydrolase, DQ269811; *Pseudomonas aurantiaca* epoxide hydrolase, BX571966; *Streptomyces avermitilis* MA-4680 α/β hydrolase, NC_003155; *Trichodesmium erythraeum* IMS101 α/β hydrolase, CP000393; *Acidothermus cellulolyticus* 11B α/β hydrolase, ZP_01136818; MonCII, thioesterase in monensin cluster, AF440781; NanE, thioesterase in nanchangmycin cluster, AF521085; NigCII, DQ354110.

the highly conserved GHSXG motif (Figure 7). The precise identities of the active site His and Asp residues of the polyether TEs are less apparent (Figure 7). The unique substrate channels that are seen in the crystal structures of both DEBS TE and PICS TE [31] appear to be long and narrow, allowing accommodation of a linear polyketide chain, while the corresponding interiors of each thioesterase consist of a flat wide cavity surrounding the catalytic triad, with a maximum diameter of 15 Å. Manual docking of the product 6-dEB into the DEBS TE cavity, followed by energy minimization, has indicated that the 14 membered macrolide can fit into this cavity without altering the protein conformation [30]. The alignment of the polyether TEs with the polyketide TEs (Figure 7) shows a stronger amino acid sequence similarity between the two groups of TEs at the N-terminal end than at the C-terminal domain, while the polyketide TEs and polyether TEs show greater intrafamily homology within their respective C-terminal domains. We speculate that these sequence differences will be reflected in differences in active site geometry and substrate specificity between the two families of thioesterases.

The hydrolysis of nanchangmycin-SNAC by NanE suggests that the natural substrate for NanE is the polyether covalently bound to the pantetheinyl side chain of NANS ACP14, rather than the parent unsaturated polyketide. According to this model, oxidative cyclization to generate the polyether would precede polyether chain release. If NanE, which is expressed in *S. nanchangensis* as a discrete protein, were able to hydrolyze unoxidized polyketide chains of variable length, one might expect to observe prematurely released products, none of which have been detected in the fermentation extracts. Importantly, *nanE* has been shown by RT-PCR (see Supplemental Data) to be cotranscribed with the discrete ACP10, *nanO*, *nanI*, and the *nanA9* PKS genes, suggesting that the thioesterase NanE, together with NanO and NanI, was coordinately coexpressed with the NANS PKS, as nonpostmodification proteins. Oxidative cyclization would therefore take place while the polyketide is still attached to the discrete ACP10.

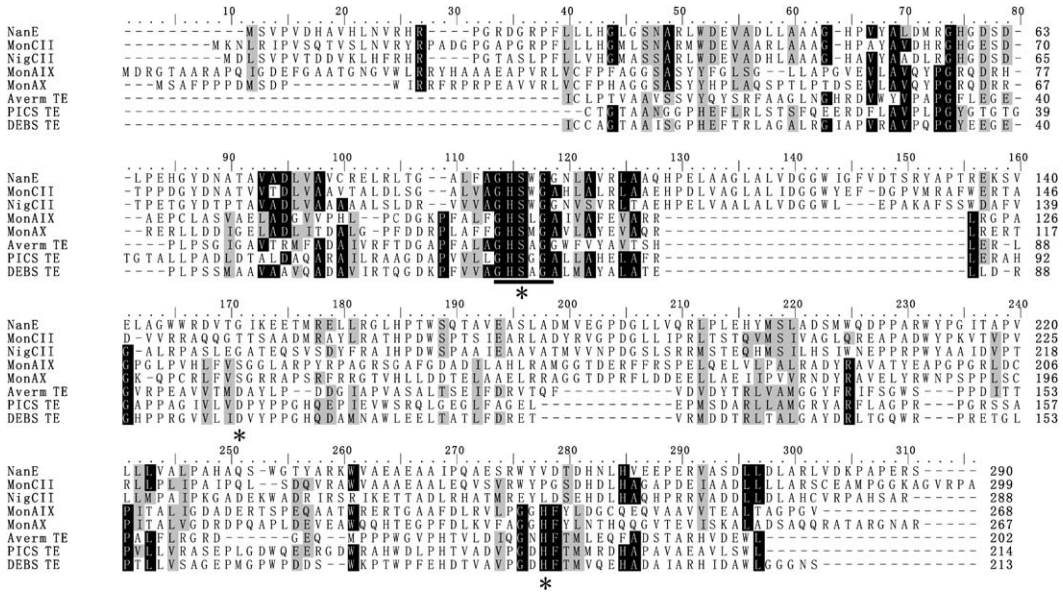

Figure 7. Multiple Alignment of NanE with Other Representative α/β Hydrolases

A conserved GHSXG motif, including the strictly conserved active site Ser is underlined in all members of the α/β hydrolyase family including NanE and type I or II TE, and the characteristic Ser, His, Asp catalytic triad of DEBS TE is highlighted by asterisks.

The in vitro hydrolysis of nanchangmycin-SNAC by recombinant NanE suggests that PKS bound nanchangmycin is the natural substrate for the thioesterase. These results do not exclude the possibility that hydrolytic release of the aglycone might precede glycosylation. Notably, however, when we analyzed a *nanG5* mutant (LTG13) generated by targeted deletion that was deficient for glycosyl transferase, the production level of the nanchangmycin aglycone was only 1% that of nanchangmycin produced by the wild-type, in which *nanG5* is constitutively expressed, as established by RT-PCR (data not shown). It is thus likely, but not certain, that NanE-catalyzed hydrolysis of PKS-linked nanchangmycin is about 100 times more efficient than hydrolytic release of the corresponding nanchangmycin aglycone from the corresponding thioester. To address this issue, additional in vitro experiments are planned for the direct determination of the substrate specificity of recombinant NanE.

Significance

The complex polyketide scaffolds of both macrolide and polyether antibiotics are each assembled on modular, multifunctional polyketide synthases. In macrolide biosynthesis, the macrolide aglycone is selectively released from the PKS by a dedicated thioesterase domain that is strategically located at the C terminus of the most downstream PKS module. Late-stage oxidations and glycosylations take place on the free macrolactone to generate the final macrolide antibiotic. By contrast, the modular PKSs that mediate the biosynthesis of polyethers such as nanchangmycin and monensin lack an evident thioesterase as an integral component of the modular PKS biosynthetic assembly line. Instead, the cascade of oxidative cyclizations of the initially generated unsaturated polyketide appears to take place prior to release of the polyether product that is mediated by a discrete thioesterase. The finding that NanE is required for nanchangmycin biosynthesis by *S. nanchangensis* NS3226, and that recombinant NanE catalyzes the specific hydrolysis of the polyether thioester analog, nanchangmycin-SNAC, establishes the identity of this chain-releasing thioesterase. Further experiments will be needed to clarify the substrate range of this specialized thioesterase and to explore the ability of this enzyme to tolerate engineered polyethers of differing chain length, stereochemistry, and substitution pattern. The high level of sequence conservation within the unique family of polyether thioesterases comprised of *nanE*, *monCII*, and *nigCII* suggests that these conserved motifs can be exploited for the design of oligonucleotide primers that could be used for the selective probing of the new polyether producers or novel gene clusters encoding the biosynthesis of the polyether compounds, thus avoiding crosshybridization with abundant, nonpolyether PKS genes that normally interferes with the use of PKS-based homology probes.

Experimental Procedures

Materials

Restriction enzymes, T4 DNA ligase, and *Pfu* polymerase were purchased from New England Biolabs. Synthesis of oligonucleotide primers and DNA sequencing of PCR products were performed by the Invitrogen Biotechnology Co., Ltd, in Shanghai. Reagents for enzyme assay and chemical synthesis were purchased from Sigma-Aldrich Chemical Co., Ltd.

Bacterial Strains, Plasmids, and Culture Techniques

S. nanchangensis NS3226 [6], the wild-type producer of nanchangmycin, was used for nanchangmycin isolation, bioassay, and the generation of mutant strains by targeted gene disruption and replacement. *E. coli* DH10B was used as cloning host, and *E. coli*

BL21(λDE3)/pLysE (Stratagene) was employed for expression of proteins. pBluescript SK(−) was used for cloning of PCR fragments, and pIJ2925 (a pUC18 derivative with double BglII sites in the polylinker [32]) was used as a transitional vector. pHZ1358 [6] (an *E. coli* and *Streptomyces* shuttle vector) was used for gene replacement in NS3226, while pIB139 [33] (a pSET152 derivative with *ermE** promoter [34] and a polylinker) was used for mutant complementation. T7-derived pET15b and pET28a were used for protein expression. GS medium was used for solid fermentation and sporulation, TSBY medium (10.3% sucrose) was used for growth of mycelium and isolation of total DNA, and SFM medium was used for conjugation. LB medium was used for *E. coli* growth and LBBS (tryptone 10 g, yeast extract 5 g NaCl 10 g, D-sorbitol 185.9 g, and betaine 0.309 g, 1 liter) for protein expression.

In-Frame Deletion of the CR Domain by Targeted Gene Replacement and Fusion of the DEBS TE and AVE TE Downstream of ACP14 or within the CR Domain

For in-frame deletion of the CR domain by targeted gene replacement, a 1706 bp DNA fragment (from cosmid 19B4 [7]) flanking the 5′-end of the CR domain was generated by digesting subclone pJTU320 with XhoI and PstI. A 1886 bp PstI-EcoRI fragment flanking the 3′-end of the CR domain was obtained by PCR amplication with a pair of primers, CR-1 (5′-AGC TCT CGA GCG GAT CGG TGG ACG G-3′, XhoI site underlined) and CR-2 (5′-CCG CCT GCA GGG CAT GTT CCA G-3′, EcoRI site underlined) followed by the digestion with XhoI and EcoRI. The two fragment arms were cloned into the PstI and EcoRI sites of pIJ2925, generating pJTU322. The 3.6 kb DNA fragment (1706 + 1886 bp) from pJTU322 was excised after BglII digestion and ligated into the BamHI site of pHZ1358 to generate a final construct pJTU323 that was introduced into strain NS3226 by conjugation from the nonmethylating *E. coli* donor strain ET12567::pUZ8002. Thiostrepton-sensitive ($Thio^S$) colonies were counter selected from the initial $Thio^R$ exconjugants after one round of nonselective growth. Three $Thio^S$ exconjugants were confirmed to be of identical deletion mutant (SL1) by PCR amplification with CR-CP1 (CP1: 5′-CGG TCA GCA GTG TCC ACA GGT C-3′) and CR-CP2 (5′-GCT GGG CTT CGA CTC GCT GA-3′) followed by further proof by Southern blotting.

For replacement of the CR domain by AVE TE, a 896 bp fragment carrying the AVE TE was obtained by PCR amplification from the total DNA of *S. avermitilis* ATCC31272 with a pair of primers, aveTE-1 (5′-GAG TCT CGA GAC CGG ACA GCA GCG-3′, XhoI site underlined) and aveTE-2 (5′-CTG ACT CGA GTT CTG TCA GC ATT C-3′, XhoI site underlined). This fragment was digested with XhoI to generate an 876 bp AVE TE fragment (292 aa) followed by cloning into the XhoI site (within the CR domain) of pJTU322, yielding pJTU327. Further cloning of the BglII-fragment from pJTU327 into the BamHI site of pJTU1358 generated pJTU331, which was used for obtaining the desired double crossover mutant (SYH30) from fifteen $Thio^S$ exconjugants, as confirmed by PCR with primers CR-CP1 and CR-CP2 and by Southern blotting.

For fusion of DEBS TE behind the SpeI site of ACP14, a 868 bp fragment carrying the DEBS TE and an engineered SpeI adaptor was obtained by PCR amplification from the total DNA of *Saccharopolyspora erythraea* with a pair of primers, TE-NdeI (5′-CAT ATG ACT AGT CAG CAG CTC GAC AGC GGG-3′, SpeI underlined) and TE-SpeI (5′-AAG ACT AGT TCA TGA ATT CCC TCC GCC CAG -3′, SpeI underlined). The left arm (1803 bp) for recombination was amplified by PCR with cosmid 19B4 as template and a pair of primers, TE-left-1 (5′-AGA AGC TTT ACA GCT GCT TGC CGG T-3′, HindIII underlined) and TE-left-2 (5′ CCA CTA GTG TGC CGT CCC TTC 3′, SpeI underlined). The right arm (1785 bp) was obtained by PCR with a pair of primers, TE-right-1 (5′-GCT CGG TAC TAG TGG CGA TCT GCT C-3′, SpeI underlined) and TE-right-2 (5′-GCG AAT TCC AGG GGG CCT TCG-3′, EcoRI underlined). The two arms were digested with SpeI and HindIII and SpeI and EcoRI, respectively, and then cloned into HindIII and EcoRI site of pIJ2925 by a ligation of three fragments, generating pJTU1380, whose DEBS TE insert was further digested with SpeI, and ligated into the SpeI site of pJTU1380, generating pJTU1381. Furthermore, the 5.4 kb DNA fragment was excised by BglII digestion and ligated into the BamHI site of pHZ1358 to generate a final construct pJTU1382 that was used for conjugation. PCR with CR-CP1 and CR-CP2 as primers identified one desired mutant (LTG18) from a total of 18 $Thio^S$ exconjugants.

Complementation of Δ*CR* Mutant by Constructs Carrying the CR Domain and/or ACP-CR Didomain

The CR domain and the ACP-CR didomain were each amplified by PCR from cosmid 19B4 with primer pairs CR-NdeI (5′-TC GCC CCG CAT ATG GAG CTG GGA CC-3′) and CR-XbaI (5′-CGG TCT AGA TCA GGA CTC GTC GTT G-3′) and ACP-CR-NdeI (5′-AC AGC TGT CAT ATG GAG GCG GTG CG-3′) and CR-XbaI (5′-CGG TCT AGA TCA GGA CTC GTC GTT G-3′), which also introduced NdeI and XbaI sites at the start codon and at the 3′ end of the gene, respectively. The PCR products of 343 bp and 569 bp, respectively, were cloned into the EcoRV site of pBluescript SK(−) and the resulting plasmids were sequenced. The CR and ACP-CR was excised from pBluescript SK(−) by digestion with NdeI and XbaI and ligated into pIB139, which places the CR domain and ACP-CR domain under the control of the P_{ermE*} promoter, generating pJTU1340 and pJTU1341, respectively. The two plasmids were each introduced into *S. nanchangensis* ΔCR (SL1) by conjugation and selection for apramycin resistance. Three expected conjugants were chosen from each group. Two pairs of primers were used for confirming the exconjungants: the pair consisting of LTG-1 (5′-GCT CAT CGG TCA GCT TCT CA-3′) and LTG-2 (5′-TCG CAT TCT TCG CAT CCC-3′) for verifying the presence of the apramycin resistance gene and the pair consisting of LTG-5 (5′-AAG GCG ATT AAG TTG GGT A-3′) and LTG-6 (5′-TGG AAT TGT GAG CGG ATA-3′) for amplifying the insert fragment cloned into the pIB139.

Targeted In-Frame Deletion of *nanE*

A 4012 bp HindIII-KpnI fragment from the sequencing subclone pJTU1361 covers the expected left arm of *nanE*, with a KpnI site internal to *nanE*. A 1825 bp fragment flanking the right side of *nanE* was amplified by PCR with *Pfu* polymerase to engineer a KpnI and a EcoRI sites at each end with primers, nanE-right2-1 (5′-G GTA CCG GAG CAG CAG GAA CG-3′) and nanE-right2-2 (5′-GAA TTC CTG TGG TCC AGT GGC-3′). The amplified PCR fragments were cloned into pBluescript SK(−) and sequenced. The right arm was excised from pBluescript SK(−) with the sites introduced by the primers and ligated with the HindIII-KpnI fragment carrying the left arm before being introduced into HindIII- and EcoRI-digested pIJ2925, to generate pJTU1363. Both arms were digested by BglII and cloned into the single BamHI site of pHZ1358, to generate plasmid pJTU1364, which was used for construction of the targeted *nanE* deletion mutant by introduction into the wild-type of NS3226 by conjugation with the same procedures as for the deletion of CR. The presence of the desired deletion in the resultant mutant strain, *S. nanchangensis* LTG10 (ΔnanE), was confirmed by PCR with primers nanE-CP1 (5′-GGC AGC CGA TAC CGA TGA-3′) and nanE-CP2 (5′-AAC GAG GAA GCC ACT CAC G-3′).

Complementation of the *ΔnanE* Mutant

The following primers were used to amplify nanE from cosmid 19B4, nanE-NdeI (5′-C CGC ATG CAT ATG TCC GTT CCG GT-3′) and nanE-XbaI (5′-TCG TTC TCT AGA TCA GGA CCG TTC C-3′) in which the two putative start sites and the NdeI and XbaI sites (underlined) are shown. The PCR fragment was cloned into pBluescript SK(−) and sequenced, and the 873 bp PCR product (NdeI-BglII digested) was then ligated into NdeI-XbaI-digested pIB139, generating pJTU1366. pUZ8002/ET12567 facilitated its subsequent conjugation and integration into *S. nanchangensis* ΔnanE, resulting in the complementation strain *S. nanchangensis* LTG11 (*ΔnanE*::*nanE*/pIB139). Similarly, the negative control strains *S. nanchangensis* LTG12 (*ΔnanE*::pIB139) and *S. nanchangensis* NS3226::pIB139 were created by using pIB139. The exconjugants were verified by PCR with primers LTG1, LTG2, LTG5, LTG6, and the same procedure as for the complementation of CR.

Construction, Expression, and Purification of Proteins Carrying the CR Domain and the ACP-CR Didomain

The CR domain and ACP-CR didomain were each amplified from cosmid 19B4 with the forward primers CR-NdeI (5′-TC GCC CCG CAT ATG GAG CTG GGA CC-3′) and ACP-CR-NdeI (5′-AC AGC TGT CAT ATG GAG GCG GTG CG-3′) and the reverse primer CR-BamHI (5′-CGG GGA TTC TCA GGA CTC GTC GTT G-3′). The forward primers introduced an NdeI restriction site (underlined), and the reverse primer introduced a BamHI restriction site (underlined).

The amplified DNA fragments were each digested with NdeI and BamHI before ligation into the expression vector pET15b. The identity of each of the resulting pET15b-CR (N-His) and pET15b-ACP-CR (N-His) constructs was confirmed by DNA sequencing. The expression vectors were separately transformed into *E. coli* BL21(DE3)/plysE cells, grown to saturation in LB medium supplemented with ampicillin (100 mg/ml) and chloramphenicol (25 mg/ml) at 37°C, and diluted 1:50 into LBBS medium supplemented with ampicillin (50 mg/ml) and chloramphenicol (12.5 mg/ml). The expression of the encoded N-terminal His_6 fusion proteins was induced at OD600 of 0.8 with 1 mM IPTG, and incubation was continued for 12 hr at 22°C.

All protein purification procedures were performed at 4°C. The cells from 1 liter cultures were harvested by centrifugation at 5000 × g for 10 min, resuspended in 25 ml of buffer A (20 mM Tris [pH 8.0], 300 mM NaCl [pH 7.5]), and lysed by ultrasonication with a Sanyo soniprep150. Cell debris was removed by ultracentrifugation (2 × 30 min at 15,000 × g), and the supernatant was loaded onto a 5 ml Hitrap chelating HP column (Amersham Biosciences). Purification of N-His_6-CR and N-His_6-ACP-CR was performed at 4°C with an ÄKTA FPLC with a flow rate of 0.5 ml/min. Absorbance was monitored at 280 nm. The column was washed with buffer A, after which the following linear gradient of buffer B (20 mM Tris [pH 8.0], 300 mM NaCl, and 500 mM imidazole [pH 7.5]) was initiated: 0%–60% B over 20 min, 60% B for 5 min, 60%–100% B over 10 min, and 100% B for 5 min. The purified protein was mixed with 20% glycerol and stored at −80°C. For the activity assay, the protein was exchanged into 50 mM phosphate buffer (pH 8.0) with a Hitrap 5 ml desalting column (Amersham Biosciences) and concentrated with a Microcon-10 concentrator (Amicon). The 50 mM phosphate buffer containing 2 mM EDTA can remove any metal ions from the native forms of the CR and ACP-CR proteins. Protein concentrations were determined by Bradford assay (Bio-Rad). Typically, 1 liter of culture yielded 20 mg of N-terminal His_6-CR and 8 mg of N-terminal His_6-ACP-CR.

Construction, Expression, and Purification of NanE, MonAX, and DEBS TE

The pET28a expression vector (Novagen) was used to express NanE, MonAX, and DEBS TE. NdeI and HindIII sites were introduced by PCR with the following primers: NanE-NdeI (5′-CC GCC ATG CAT ATG TCC GTT CCG GT-3′) and NanE-HindIII (5′-TCG TTC AAG CTT TCA GGA CCG TTC C-3′), MonAX-NdeI (5′-CAT ATG TCT GCC TTC CCC CCA CCC G-3′) and MonAX-HindIII (5′-AAG CTT TCA CCG AGC GTT CCC CCT T-3′), and TE-NdeI (5′-CAT ATG *ACT AGT* CAG CAG CTC GAC AGC GGG-3′) and TE-HindIII (5′-CGG AAG CTT TCA TGA ATT CCC TCC GCC CAG-3′). The respective three templates were cosmid 19B4, total DNA of *S. cinnamonensis*, and total DNA of *Saccharopolyspora erythraea*. Each of the three indivdual PCR products was sequenced after cloning into pBluescript SK(−). The clones containing the three genes were then digested with NdeI-HindIII and the desired DNA fragments were ligated into the NdeI-HindIII sites of pET28a, generating pJTU1373, pJTU1374, and pJTU1375, respectively. The N terminus of the recombinant DEBS TE, which started from Gln-Gln-Leu within the linker between ACP6 and TE, had a predicted MW of 32,489.23 Da. Plasmids pJTU1373, pJTU1374, and pJTU1375 were transformed into *E. coli* BL21 (DE3)/pLysE for protein expression. Kanamycin (25 mg/ml) and chloramphenicol (12.5 mg/ml) were used in the LB medium for maintaining the plasmids. The three resultant proteins all carried an N-terminal His_6 tag. The expression and purification protocols, which were the same for all three proteins, were the same as those used for the CR and ACP-CR domains, with only a modification of the FPLC conditions were different. The linear gradient of buffer B (20 mM Tris [pH 8.0], 300 mM NaCl, and 500 mM imidazole [pH 7.5]) was initiated: 0%–100% B over 30 min and 100% B for 5 min with a flow rate of 0.5 ml/min. All three proteins were stable at 4°C as a 10 mg/ml stock solution (50 mM phosphate buffer [pH 8.0]).

Protein Characterizations by MALDI-TOF-MS

The N-terminal His-tagged NanE and and CR domain proteins were exchanged from 50 mM to 5 mM phosphate buffer, and the protein concentration was adjusted to 1 mg/ml. Analysis was performed by using MALDI-TOF mass spectrometer (Bruker) and 337 nm pulsed (4 ns) N_2 laser operated in linear mode. All mass spectra were obtained in positive ion mode with a 20 kV source voltage and a flight distance of 1.5 m. The observed MW of CR protein was 13281.6, and its dimer with a MW of 26570.0445 was also observed. The MW of NanE was measured as 33933.7.

(2*S*,3*R*)-2-methyl-3-hydroxypentanoyl *N*-acetylcysteamine Thioester

(2*S*,3*R*)-2-Methyl-3-hydroxypentanoyl-SNAC was prepared as previously described [35, 36].

Nanchangmycin-SNAC

Nanchangmycin A (70 mg) was prepared by preparative RP-HPLC from 8 liter of solid culture of *S. nanchangensis* NS3226. To a solution of nanchangmycin A (64 mg), EDC•HCl (48 mg), and DMAP (2.5 mg) in CH_2Cl_2 (5 ml) was added *N*-acetylcysteamine (65 μl), and the mixture was shaken at 100 r/min for 16 hr at 23°C. The reaction mixture was partitioned between 0.1 N aqueous HCl (5 ml) and EtOAc (5 ml). The aqueous layer was extracted with EtOAc (25 ml), and the organic extracts were washed with saturated NaCl (5 ml), dried (Na_2SO_4), and concentrated under reduced pressure to give a colorless oil [37]. The resulting oil was dissolved in 100% methanol (10 ml) and purified by preparative reverse-phase HPLC (VP-ODS 20 × 100 mm, SHIMADZU). Isocratic elution of 90:10 CH_3OH:H_2O at a flow rate of 5 ml/min monitoring at 230 nm afforded 10 mg nanchangmycin-SNAC (t_R = 12.0 min). The combined fractions were concentrated under reduced pressure after drying with Na_2SO_4. The efficiency of reaction was almost 100% when the system was allowed to mix thoroughly by shaking, as was estimated by the complete absence of nanchangmycin in the HPLC of the product mixture at the end of the reaction, although losses upon sample purification were encountered. The nanchangmycin-SNAC was analyzed by ESI-MS, *m/z* 990.5 ($C_{51}H_{85}NO_{14}S$ + Na^+ requires 990.57), and ESI LC-MS/MS and ESI LC-MS^3 comparison with the mass spectrum of nanchangmycin A ($C_{47}H_{78}O_{14}$+ Na^+ requires 889.54). The structure of nanchangmycin-SNAC was further confirmed by the ^1H and ^{13}C NMR (500 MHz, Bruker) (see Supplemental Data for details).

Hydrolytic Activity Assay toward SNAC Substrates

Hydrolysis of diketide-SNAC substrates was followed spectrophotometrically by reaction of released HSNAC with 5,5′-dithio-2-nitrobenzoic acid (DTNB) and monitoring of the formation of 5-thio-2-nitrobenzoate (λ_{max} = 412 nm, ε = 13 600 $M^{-1}cm^{-1}$). To determine which protein could hydrolyze the diketide-SNAC, each assay mixture contained 50 mM phosphate buffer (pH 8.0), 10 μM protein, 5 mM substrate, and 6% (v/v) DMSO in a total volume of 500 μl. The reaction mixture was incubated at 30°C. At intervals of 3, 7, 15, 30, 45, and 60 min, 100 μl samples were withdrawn and the reaction was quenched by mixing with 20 μl of 1 M HCl. The protein was removed with a 10,000 Micropure-EZ (Amicon) ultrafiltration unit. The amount of released free thiol was quantitated at 412 nm by mixing with 150 μl of a saturated solution of DTNB in 50 mM phosphate buffer (pH 8.0) and 730 μl of 50 mM phosphate buffer (pH 8.0) [26]. The stock solution of diketide-SNAC was 250 mM in DMSO. In all cases, the measured absorbance was corrected for the background rate of chemical hydrolysis in the absence of enzyme.

Hydrolysis of polyether-SNAC was also monitored by spectrometric observation of the formation of 5-thio-2-nitrobenzoate at (λ_{max} = 412 nm, ε = 13 600 $M^{-1}cm^{-1}$) as well as by more direct HPLC monitoring at 230 nm of the formation of nanchangmycin. To determine which protein could hydrolyze nanchangmycin-SNAC, the assay mixture contained 50 mM phosphate buffer (pH 8.0), 10 μM protein, 38.4 μM substrate, 5% methanol in a total volume of 5 ml. The reaction mixture was incubated at 30°C. At intervals of 3 min, 7 min, 15 min, 30 min, 45 min, 60 min, 90 min, and 2 hr, 200 μl samples were removed from the reaction system and extracted with 500 μl EtOAc. The organic extracts were concentrated under reduced pressure and redissolved in methanol. The liquid phase was analyzed by LC-MS/MS to detect the conversion of nanchangmycin-SNAC to nanchangmycin. t_R in LC: HSNAC, 1.3 min; nanchangmycin-SNAC, 15.6 min; nanchangmycin, 17 min.

Extraction of Antibiotics and LC-MS Analysis

Extracts of wild-type and mutant strains of *S. nanchangensis* were prepared by overnight extraction of 10-day-old solid fermentation

cultures with two volumes of methanol, followed by two rounds of centrifugation at 15,000 × g for 10 min. The supernatants were concentrated under reduced pressure, and the residue was redissolved in methanol. The crude extracts were analyzed by ESI LC-MS/MS (Agilent 1100 series LC/MSD Trap system) with an Agilent Extend C-18 (2.1×150 mm, 5 nm) column. The LC was operated at a rate of 0.3 ml/min at room temperature. Eluent A was Milli-Q deionized water with 0.1% TFA, and eluent B was methanol (Merck) with 0.1% TFA. The HPLC conditions were 0.01–8.00 min 85% B, 8.00–13.00 min 95% B, 13.00–23.00 min 100% B, and 23.00–28.00 min 100% B. The mass spectrometer was run in positive ion detection mode and set to scan between 50 and 1500 *m/z*. Drying gas flow was 10 l/min, 325°C, and a nubulizer pressure of 30 psi.

Supplemental Data

Supplemental Data for the determinations of the cotranscription involving *nanE* by RT-PCR analysis and the structure of nanchangmycin-SNAC by MS and NMR are available at http://www.chembiol.com/cgi/content/full/13/9/945/DC1/.

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