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An Iterative Type I Polyketide Synthase PKSN Catalyzes Synthesis of the Decaketide Alternapyrone with Regio-Specific Octa-Methylation

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Summary

A biosynthetic gene cluster containing five genes, *alt1–5*, was cloned from *Alternaria solani*, a causal fungus of early blight disease to tomato and potato. Homology searching indicated that the *alt1*, *2*, and *3* genes code for cytochrome P450s and the *alt4* gene for a FAD-dependent oxygenase/oxidase. The *alt5* gene encodes a polyketide synthase (PKS), named PKSN, that was found to be an iterative type I complex reduced-type PKS with a *C*-methyltransferase domain. To identify the PKSN function, the *alt5* gene was introduced into the fungal host *Aspergillus oryzae* under an α-amylase promoter. The transformant produced a polyketide compound, named alternapyrone, whose structure is shown to be 3,5-dimethyl-4-hydroxy-6-(1,3,5,7,11,13-hexamethyl-3,5,11-pentadecatrienyl)-pyran-2-one. Labeling experiments confirmed that alternapyrone is a decaketide with octa-methylation from methionine on every C_2 unit except the third unit.

Introduction

Filamentous fungi produce a wide range of natural products, including β-lactam antibiotics (e.g., penicillin), cholesterol-lowering statins (e.g., lovastatin), and mycotoxins (e.g., aflatoxins). Many of these are polyketides, a large group of secondary metabolites which display huge structural diversity. Polyketide carbon skeletons are biosynthesized by so-called polyketide synthases (PKSs), which join short-chain carboxylic acid units in a stepwise fashion, similarly to fatty acid synthetases. All PKSs so far found in fungi are type I irrespective of whether their product compounds belong to the aromatic or reduced complex-type groups [1, 2]. In contrast to bacterial type I PKSs which have a multimodular organization, fungal PKSs have a single modular architecture with linear organization of active site domains similar to that of mammalian fatty acid synthetases. Thus, they are classified as iterative type I PKSs. In addition to β-ketoacyl synthase (KS), acyltransferase (AT), and acyl carrier protein (ACP), which are the fundamental domains in all type I PKSs, auxiliary functional domains have often been identified in fungal PKSs. To modify β-carbonyls in the synthesis of complex reduced-type compounds, fungal PKSs utilize β-ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains. Methyltransferase (MeT) domains have been identified in some fungal PKSs, such as *Aspergillus terreus* LNKS and LDKS for lovastatin synthesis in which they serve as *C*-methyltransferases (CMeTs) [3]. In addition, we recently reported the Claisen cyclase domain in fungal aromatic PKSs such as *Aspergillus nidulans* WA [4]. During assembly of the polyketide-derived skeleton by fungal PKSs, these auxiliary domains are not involved in every condensation cycle but participate selectively in specific cycles in a predetermined manner that leads to a variety of functionality on the polyketide chains, especially in complex reduced-type compounds. However, how these iterative type I fungal PKSs control the exact sequence of their reactions, that is, the number of condensation cycles, extent of carbonyl reduction, and degree of methylation, polyketide chain folding, and cyclization, and so forth, remains largely unknown.

Alternaria solani is a causative agent of early blight disease to tomato and potato. The fungus produces various structurally unrelated polyketide metabolites, both aromatic and complex reduced-type compounds [5] (Figure 1). Of these, alternaric acid was isolated as an antifungal metabolite in 1949 by Brian and coworkers [6–8]. This compound was shown to contribute to disease development in the plant host by the fungus [9]. Feeding experiments with isotopically labeled acetate indicated that alternaric acid is biosynthesized from two polyketide chains rather than a single chain [5]. *A. solani* also produces other complex reduced-type polyketides, the solanapyrones [10–12]. Biosynthetic studies on the solanapyrones strongly suggested that a biological Diels-Alder reaction is involved in formation of their decalin skeleton from a precursor octaketide α-pyrone, prosolanapyrone [13–15]. Although intensive enzymological studies on the Diels-Alderase have been carried out, characterization of the enzyme has faced difficulties mainly due to its instability. In contrast to the independent Diels-Alderase in solanapyrone biosynthesis, the lovastatin nonaketide synthase (LNKS) was itself shown to catalyze a Diels-Alder cycloaddition [16]. Recently, Schmidt and coworkers identified a PKS-NRPS (nonribosomal peptide synthase) gene involved in equisetin biosynthesis in *Fusarium heterosporum* and suggested that the similar carbocyclic skeleton of equisetin is synthesized by the PKS part of the EqiS PKS-NRPS enzyme [17]. However, it is still not known how these PKSs catalyze and control Diels-Alderase activities.

To examine interesting features of polyketide biosynthesis in *A. solani*, in particular biosynthesis of the complex reduced-type compounds, we adopted a molecular genetic methodology targeting the PKS genes. The highly conserved regions within the functional domains are logical targets for the detection of PKS genes in fungi. Primer pairs have been developed for the KS, KR, and CMeT domains of fungal PKSs and are successfully

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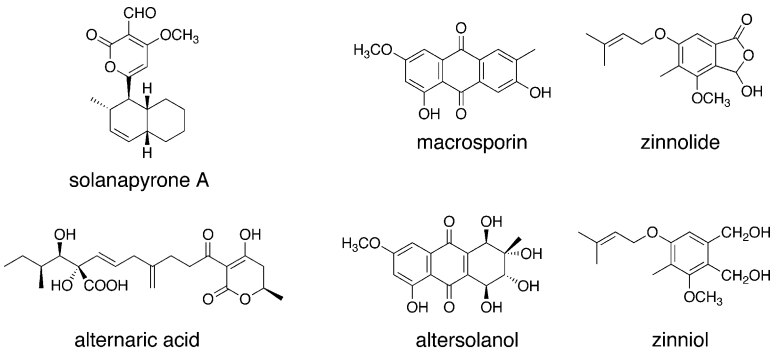


Figure 1. Structures of Metabolites Isolated from *Alternaria solani*

used for the cloning of fungal complex reduced-type PKS (RD-PKS) genes [18]. The fumonisin [19] and compactin [20] PKS genes have been cloned. In addition, Cox and coworkers reported the cloning of an RD-PKS gene involved in squalestatin biosynthesis [21] and an RD-PKS-NRPS gene involved in fusarin biosynthesis in *Fusarium moniliforme* [22]. Schmidt and coworkers used a similar strategy for cloning the *eqiS* gene and *eqi* gene cluster from *F. heterosporum* [17].

We designed a new degenerate primer pair for amplification of fungal PKS genes and used it to obtain a PKS gene fragment from *A. solani*. Using this fragment as a screening probe, we cloned a gene cluster consisting of genes for three cytochrome P450s, one FAD-dependent oxygenase/oxidase, and one PKS from *A. solani*. These have been designated *alt1*, *2*, *3*, *4*, and *5*, respectively. Functional expression of the *alt5* gene encoding a PKS named PKSN in a heterologous host, *Aspergillus oryzae*, gave production of a new, to our knowledge, decaketide compound, alternapyrone, which has eight methyl side chains derived from methionine. Although PKSN is not the PKS for solanapyrones or alternaric acid, it is, to our knowledge, the first iterative type I fungal PKS identified to catalyze regio-specific octamethylation during polyketide synthesis.

Results and Discussion

Amplification of β-Ketoacyl Synthase Regions of *A. solani* PKS Genes

To clone fungal RD-PKS genes, a pair of degenerate primers was designed from the conserved regions of known fungal RD-PKS genes, such as LNKS of *Aspergillus terreus* [3] and PKS1 of *Cochliobolus heterostrophus* [23]. Amino acid sequence alignment of these fungal RD-PKSs indicated a highly conserved region around the KS domains. The conserved amino acid sequences E-C/A-H-G-T-G-T and G-Q-G-A-Q-W located between the KS and AT active sites were chosen to design the forward and reverse primers, KSD-F and KSD-R, respectively. PCR amplification from the *A. solani* genomic DNA gave an approximately 900 bp long fragment, which was subsequently cloned into pT7-Blue T vector. From the clones sequenced, two different KS fragments were obtained. One showed high homology with fungal RD-PKSs such as LDKS [3] and FUM1p [19], and the other showed homology with fungal aromatic PKSs. The former PCR fragment was temporarily named *pksA*.

Cloning of an RD-PKS Gene Cluster from *A. solani*

An initial *A. solani* genomic DNA library was constructed in pANsCos1. A positive clone, designated pAN1, was obtained from the library by screening with the digoxigenin (DIG)-labeled *pksA* probe. Restriction enzyme digestion and Southern blot analysis indicated that its insert was around 12 kb long, which was much smaller than the insert size of 30–40 kb expected for this cosmid vector. This is probably due to deletion during plasmid propagation. Sequencing of pAN1 showed that the *pksA* fragment was located at the end of the pAN1 insert with its upstream region, about 11 kb, containing some biosynthetic open reading frames (ORFs). Because other positive clones also had smaller inserts than expected, a second genomic DNA library was constructed in pOJ446 which has been used successfully for construction of actinomycete genomic DNA libraries [24].

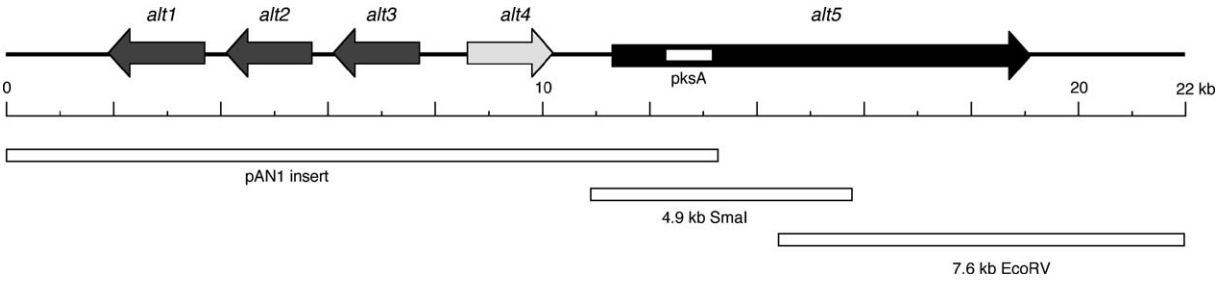
A positive clone, pJ18, obtained from the second library, was chosen for further analysis. A 4.9 kb SmaI fragment from the pJ18 insert hybridized with the *pksA* probe, and this and a 7.6 kb EcoRV fragment of the pJ18 insert overlapping with the SmaI 4.9 kb fragment were sequenced. These, together with sequencing of the pAN1 insert, gave the nucleotide sequence of a 22 kb region in the pJ18 insert. Frame plot analysis indicated the presence of five ORFs in this region, which encoded three cytochrome P450s, one FAD-dependent oxygenase/oxidase, and one PKS. These genes were named *alt1*, *2*, *3*, *4*, and *5*, respectively (Table 1). No other possible biosynthetic ORFs were found in either the upstream or downstream regions adjacent to the *alt* gene cluster.

Architecture of PKSN

Alt5 is a 7832 bp long gene deduced to encode a 2551 amino acid PKS, named PKSN. The presence of three introns (positions 186–245, 805–870, and 1257–1306 from the start codon) was confirmed by reverse transcription (RT)-PCR and the sequence analysis of *alt5* cDNA obtained from the PKSN expression transformant *A. oryzae*/pTA-PKSN10a described below. Catalytic domain searching indicated that PKSN is a multifunctional iterative type I RD-PKS with KS, AT, DH, MeT, KR, ER, and ACP domains. PKSN showed high similarity to other fungal RD-PKSs, and in particular shares 35% identity with LDKS (LovF), which is involved in lovastatin biosynthesis [3]. In particular, the ER domain of PKSN showed 44% identity with that of LDKS but no significant similarity to that of LNKS (LovB), which is known to be nonfunctional and require the independent ER enzyme LovC [3].

Table 1. The *alt* Gene Cluster from *Alternaria solani* and Deduced Functions of ORFs

Gene	Amino Acids (bp)	Protein Homolog, Origin (Accession Number, DDBJ/GenBank/EMBL)	Proposed Function
alt1	524 (1804)	AvnA, *Aspergillus nomius* (AAS66008)	Cytochrome P450
alt2	537 (1614)	ALK1, *Yarrow lipolytica* (BAA31433)	Cytochrome P450
alt3	509 (1650)	AvnA, *Aspergillus flavus* (AAS90010)	Cytochrome P450
alt4	482 (1449)	EncM, *Streptomyces maritimus* (AAF81732)	FAD-dependent oxygenase/oxidase
alt5	2551 (7832)	LDKS, *Aspergillus terreus* (AAD34559)	Polyketide synthase



A MeT domain with *S*-adenosyl-L-methionine (AdoMet) binding motifs was found in the center of the PKSN protein. Because *O*- and *N*-methylations are catalyzed by enzymes separate from PKSs in fungal polyketide biosynthesis, the MeT domain of PKSN could be a CMeT catalyzing *C*-methylation during polyketide synthesis. The deduced architecture of PKSN is shown schematically in Figure 2A together with other fungal RD-PKSs.

Expression of PKSN in *Aspergillus oryzae*

The complex reduced-type polyketides produced by *A. solani* reported so far are alternaric acid and the solanapyrones [5]. These compounds both contain C_1 substituents derived from methionine. Thus, the product of PKSN was expected to be one of their precursors or a related compound. To identify the actual function of PKSN, expression of the *alt5* gene was carried out in the *A. oryzae* fungal expression system using an α-amylase promoter [25]. The PKSN expression plasmid pTA-PKSN based on the expression vector pTAex3R was constructed by GATEWAY cloning technology (Invitrogen, Carlsbad, CA) and introduced into the host fungus *A. oryzae* by protoplast-polyethylene glycol transformation [26]. Ten pTA-PKSN transformants obtained were then subjected to induction culture to analyze their production. Although no product could be detected in the induction culture media of all transformants, mycelial acetone extracts of the induction cultures of seven transformants showed the production of a compound which was not present in either the control transformant with pTAex3R or untransformed *A. oryzae* M-2-3. For the product and functional analysis of PKSN, one of the transformants, designated *A. oryzae*/pTA-PKSN10a, was chosen, in which mRNA for the whole PKSN coding region was confirmed by RT-PCR and expression of the full-size PKSN protein was detected by SDS-PAGE (Figure 2B).

Structure Determination of Alternapyrone

HPLC analysis of the mycelial extracts of *A. oryzae*/pTA-PKSN10a indicated that the peak at Rt 13.3 min is the sole product of the transformant and LC-APCIMS analysis gave *m/z* 429 $[M+H]^+$ and 427 $[M-H]^-$. Thus, the molecular weight of the PKSN product was found to be 428 (Figure 3).

To determine the chemical structure of the product, 30 g fresh weight mycelia from the induction-cultured *A. oryzae*/pTA-PKSN10a was extracted with acetone at room temperature overnight. The extract was concentrated in vacuo and reextracted with ethyl acetate. Evaporation of ethyl acetate gave 35 mg of an oily residue. One half of the residue was dissolved in chloroform and applied onto a silica gel column (Wako gel C-200, 2 × 20 cm; Wako, Osaka, Japan). Elution with chloroform gave the PKSN product (15 mg) as a colorless oil.

The molecular formula of the PKSN product was determined to be $C_{28}H_{45}O_3$ by high-resolution EI-MS ($[M]^+$ *m/z* 428.3302 [428.3282 calculated for $C_{28}H_{44}O_3$]). From ^{13}C and ^1H NMR data (Table 2) and 2D-NMR data including ^1H-^1H COSY, HMQC, and HMBC (see Supplemental Data available with this article online), the chemical structure of the PKSN product was determined to be 3,5-dimethyl-4-hydroxy-6-(1,3,5,7,11,13-hexamethyl-3,5,11-pentadecatrienyl)-pyran-2-one. This compound was named alternapyrone (Table 2).

Alternapyrone Synthesis by PKSN

The structure of alternapyrone indicated that it was a decaketide-derived α-pyrone with eight methyl branches. To identify the origins of the alternapyrone carbons, feeding experiments with sodium [1,2-$^{13}C_2$]acetate and [methyl-^{13}C]methionine were carried out. From the ^1JC-C couplings and 2D-INADEQUATE data of alternapyrone labeled with [1,2-$^{13}C_2$]acetate, a linear decaketide backbone was identified as expected. Incorporation of methionine into all eight methyl branches was confirmed (Supplemental Data).

Several of the RD-PKSs so far cloned possess a CMeT domain which is located in the center of their domain organization (Figure 2A). The CMeT domain of LNKS is involved in the PKS-catalyzed chain assembly sequence once only in the third condensation cycle to form

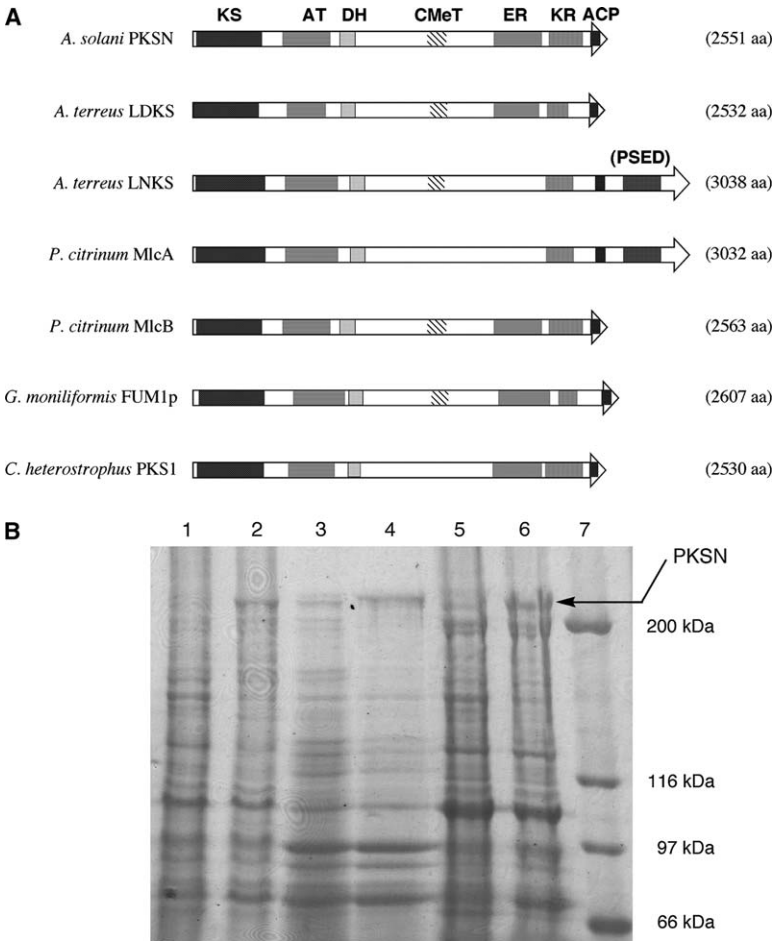


Figure 2. RD-PKS Architecture and SDS-PAGE Analysis of *A. oryzae* Transformants

(A) Architecture of the PKSN polyketide synthase from *Alternaria solani* and other fungal reduced complex-type polyketide synthases. Schematic organization of domains β-ketoacyl synthase (KS), acyltransferase (AT), dehydratase (DH), *C*-methyltransferase (CMeT), enoyl reductase (ER), β-ketoreductase (KR), and acyl carrier protein (ACP) of PKSN, FUM1p (AAD43562, *Gibberella moniliformis*), LDKS (AAD34559, *Aspergillus terrues*), LNKS (AAD39830, *Aspergillus terreus*), MlcA (BAC20564, *Penicillium citrinum*), MlcB (BAC20566, *Penicillium citrinum*), and PKS1 (AAB08104, *Cochliobolus heterostrophus*). PSED is a homolog of a peptide synthase elongation domain.

(B) Heterologous expression of PKSN. SDS-PAGE analysis: lane 1, 100 k *g* precipitate of *A. oryzae*/pTAex3R extract; lane 2, 100 k *g* precipitate of *A. oryzae*/pTA-PKSN10a extract; lane 3, 210 k *g* supernatant of *A. oryzae*/pTAex3R extract; lane 4, 210 k *g* supernatant of *A. oryzae*/pTA-PKSN10a extract; lane 5, 210 k *g* precipitate of *A. oryzae*/pTAex3R extract; lane 6, 210 k *g* precipitate of *A. oryzae*/pTA-PKSN10a extract; and lane 7, molecular weight markers.

a methylated tetraketide intermediate [3]. In contrast, the CMeT domain of PKSN is apparently involved in every condensation cycle except the third condensation. Nucleophilic attack on the methyl group of AdoMet by the active methylene formed by the ketosynthase-catalyzed condensation reaction is considered to be chemically reasonable. Thus, the stepwise reaction scheme for alternapyrone synthesis by PKSN shown in Figure 4 can be proposed.

Heterologous expression of LNKS in *A. nidulans* resulted in the production of polyene pyrone compounds rather than the expected reduced precursor of lovastatin, and it was shown that this "malfunctioning" was compensated by coexpression of a separate accessory enoylreductase encoded by the *lovC* gene [3]. In marked contrast, all the domains in PKSN are apparently involved in alternapyrone synthesis, that is, the eight CMeT, seven KR, seven DH, and four ER reactions in the nine KS-mediated condensation steps required for alternapyrone synthesis. No ORF of a separate accessory function for PKSN was found in the *alt* gene cluster and its flanking region. Interestingly, the PKS reaction scheme for the alternaric acid main chain is assumed to follow the PKSN reaction until the fourth condensation cycle, at least as far as the *C*-methylation pattern is concerned. However, the *A. solani* strain used in this study produces no alternaric acid. Thus, alternapyrone must be considered to be the correct product of PKSN and not a malformed or shunt product of the PKS for solanapyrone or alternaric acid.

Methyltransferase Motifs

AdoMet-dependent methyltransferases (MTases) are abundant and methylate a wide variety of molecular targets, including DNA, RNA, proteins, and small molecules. MTases catalyze methyltransfer from AdoMet to either nitrogen, oxygen, or carbon atoms of their substrate compounds specifically. In spite of the wide variety of methyl acceptor substrates and their low overall sequence identity to each other, AdoMet-dependent MTases contain conserved motifs which are considered to be AdoMet binding regions. Kagan and Clarke reported widespread occurrence of three conserved sequence motifs, I, II, and III, in AdoMet-dependent MTases [27]. In multifunctional enzymes such as type I PKSs and NRPS, the presence of MeT domains is usually deduced on the basis of conservation of these motifs and homology with known MTases and MeT domains. Fauman et al. [28] redefined AdoMet binding motif nomenclature, identifying motifs I–VII, in which motifs I and IV correspond to Kagan and Clarke motifs I and II, respectively. Mapping and molecular modeling of AdoMet binding sites in *N*-MeT domains of cyclosporin synthase supported the idea that the four noncontiguous motifs I–IV govern interaction with the methyl donor AdoMet [29]. From the crystal structures of the MTase

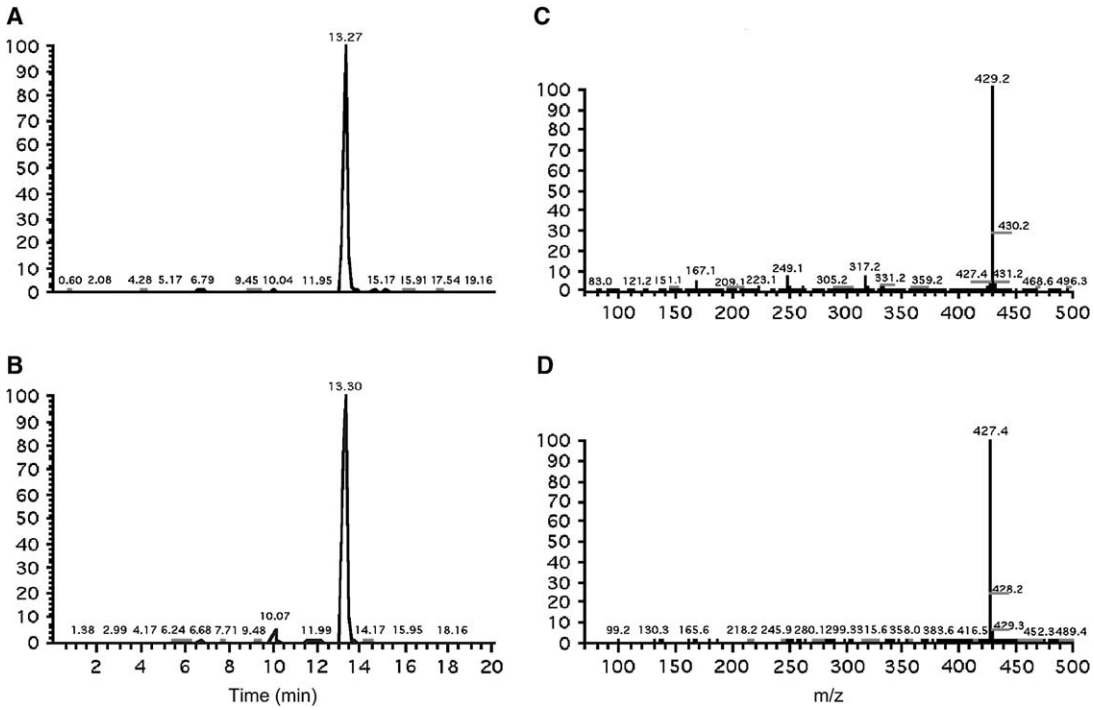


Figure 3. LC-MS Analysis of Product from the Transformant *Aspergillus oryzae*/pTA-PKSN10a

(A) Total positive ion chromatogram.
(B) Total negative ion chromatogram.
(C) Mass spectrum of the peak Rt = 13.3 min in (A).
(D) Mass spectrum of the peak Rt = 13.3 min in (B).

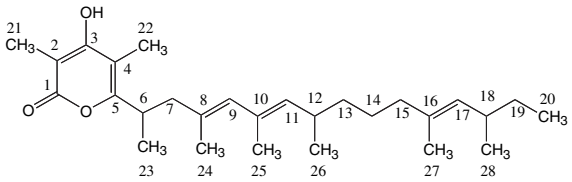
from *Thermotoga maritima* 0872 (TM0872) with both AdoMet and *S*-adenosyl-L-homocysteine (AdoHcy), Anderson and coworkers identified four AdoMet binding motifs that have spatial and functional homology with other MTases [30]. Anderson's motif I is a glycine-rich region that forms the binding pocket surrounding the L-homocysteine portion of AdoHcy, and is commonly defined as motif I by Kagan and Clarke, and Fauman et al. The key amino acid residue in Anderson's motif II is aspartic acid or glutamic acid, which interacts with the ribose 2′ and 3′ hydroxyls of AdoMet. Next to the acidic amino acid is a bulky aliphatic amino acid such as leucine, proline, or valine, which is in van der Waals contact with the AdoMet adenine ring. This motif II corresponds to that defined by Fauman et al. An aromatic amino acid, tyrosine or phenylalanine, present in Anderson's motif III, which corresponds to the Kagan and Clarke motif II and motif IV of Fauman et al., binds to the AdoMet adenine ring by π-π stacking interaction. Aspartic acid or glutamic acid in Anderson's motif IV, which corresponds to Kagan and Clarke's motif III, interacts with the amino group of AdoHcy. Conservation of these four motifs was also confirmed in caffeic acid/5-hydroxyferulic acid 3/5-O-methyltransferase from alfalfa, whose crystal structure has also been determined [31].

In fungal iterative type I RD-PKSs, these AdoMet binding motifs are found in the central region of the PKS polypeptides. Interestingly, evidence for these motifs is also found in *Cochliobolus heterostrophus* PKS1 for T-toxin synthesis [23] and MlcA for compactin nonaketide synthesis [20], although their products are apparently nonmethylated polyketides. Following the definition by Anderson and coworkers [30], amino acid sequence alignment of CMeT domains in iterative type I PKSs is shown in Figure 5. Critical mutations seen in motifs I and IV for MlcA and in motif I for PKS1 might be the reason for production of nonmethylated polyketides, and this should be proved experimentally. On the other hand, how regio-specific methylation of the polyketide intermediate is controlled during RD-PKS-catalyzed chain extension is not understood.

Several linear α-pyrone-containing polyketide fungal metabolites are known, such as citreomontanin [32], citreoviridin [33, 34], asteltoxin [35], and so forth. These are all α-pyrones substituted with multimethylated alkyl chains. From *Aspergillus niger* FT-0554, naphredin was isolated as a novel inhibitor of NADPH-fumarate reductase [36]. This compound is also an open chain nonaketide δ-lactone with a methylated olefinic side chain. The origin of its branched methyl groups was determined to be methionine by feeding experiments. Thus, in the biosynthesis of these multimethylated open chain fungal polyketides, PKSs similar to PKSN could be operative for their initial biosynthetic steps to elaborate their carbon skeletons. PKSN encoded by *alt5* is, to our knowledge, the first PKS to be identified for biosynthesis of this class of compounds. Recently, Cox et al. reported cloning and expression of the *Phoma* PKS1 gene encoding SQTKS (squalestatin tetraketide synthase) and identified a doubly methylated unsaturated acid as its product [21]. The PKS region of the *FusA* gene appears to be responsible for the synthesis of a tetramethylated heptaketide intermediate in fusarin biosynthesis [22]. Further functional analysis of these RD-PKSs is necessary to

Table 2. ^{1}H and ^{13}C NMR Spectral Data for Alternapyrone

Position Number	δ ^{13}C (mult.)[a]	δ ^{1}H (int., mult., *J* in Hz)[b]
1	166.2	—
2	97.9	—
3	164.9	—
4	106.3	—
5	161.5	—
6	33.3	3.04 (1H, m)
7	45.0	2.31 (1H, m), 2.16 (1H, m)
8	131.8	—
9	131.6	5.52 (1H, s)
10	130.7	—
11	136.0	4.85 (1H, d, 9.5)
12	32.3	2.31 (1H, m)
13	37.0	1.14 (2H, m)
14	25.7	1.30 (2H, m)
15	39.7	1.89 (2H, m)
16	133.5	—
17	131.1	4.81 (1H, d, 9.3)
18	33.9	2.16 (1H, m)
19	30.5	1.14 (2H, m)
20	11.9	0.77 (3H, t, 7.4)
21	8.5	1.97 (3H, s)
22	9.5	1.92 (3H, s)
23	17.6	1.14 (3H, m)
24	17.6	1.66 (3H, s)
25	17.0	1.57 (3H, s)
26	15.9	0.88 (3H, d, 6.6)
27	15.9	1.52 (3H, s)
28	20.9	0.85 (3H, d, 6.6)



[a] Recorded at 125 MHz.
[b] Recorded at 500 MHz. Solvent: $CDCl_3$.

understand the details of their mechanistic aspects including how they control polyketide chain length, β-carbonyl reduction, and regio-specific methylation of α-carbons.

The main reduced complex-type compounds produced by the *A. solani* strain used in this study are the solanapyrones [10]. We had expected to clone the gene encoding the PKS responsible for prosolanapyrone. However, no KS fragment of an RD-PKS other than that of PKSN was amplified by the PCR conditions used in this study. Recent fungal genomic studies revealed the presence of a number of PKS genes in the genomes of fungi such as *Aspergillus nidulans* (*Aspergillus* Sequencing Project; Broad Institute of MIT and Harvard, http://www.broad.mit.edu), *Magnaporthe grisea* [37], and *Neurospora crassa* [38]. Cloning of other RD-PKS genes including a gene for prosolanapyrone synthesis is now being attempted with newly designed PCR primers in our laboratory. It is noteworthy that alternapyrone and/or its derivatives have not been detected in *A. solani* thus far. Because transcription of *alt5* mRNA was confirmed by RT-PCR (data not shown), the alternapyrone formed might be intensively modified by cytochrome P450s and FAD-dependent oxygenase/oxidase in the *alt* gene cluster. Thus, to identify the overall function of the *alt* gene cluster, coexpression of *alt1–4* genes with *alt5* is now underway in our laboratory.

Significance

Fungal polyketides have been a rich source of biologically active compounds. Their basic carbon skeletons are constructed by iterative type I PKSs. Sequence information on fungal PKSs has mainly been increasing due to fungal genome projects. However, there have been a few examples in which the actual functions of fungal PKSs have been identified by expression and product identification in either aromatic or complex reduced-type PKSs. Because active site domains are highly conserved in fungal PKSs, annotation has become easier for PKS genes. However, it is still not possible to assess their overall functions and deduce the details of the carbon skeleton that will be produced by each PKS from sequence information alone. The *alt* gene cluster containing an RD-PKS gene, *alt5*, was cloned by homology-based PCR and genomic DNA library screening from *A. solani*, which produces structurally and biosynthetically interesting complex reduced-type polyketides such as solanapyrones. The role of PKSN was identified to be a synthase responsible for production of an entirely new, to our knowledge, compound alternapyrone, which has not been detected in *A. solani*., Feeding experiments confirmed that alternapyrone is a decaketide with eight methyl side chains, derived from methionine, which could be introduced by the CMeT domain of PKSN. The presence of CMeT domains in fungal RD-PKSs is not uncommon, but the PKSs with CMeT domains identified so far catalyze only single, di-, or tetramethylation. PKSN is thus, to our knowledge, the first iterative type I PKS identified that introduces multiple methyl side chains on almost all of the α-carbons after ketosynthase reactions in a condensation cycle-specific manner. It is still unknown how iterative type I PKSs control their reactions, such as the number of condensations and degree of reduction, *C*-methylation, and cyclization. Further functional analysis on PKSN will help to solve these critical aspects of iterative type I PKSs.

Experimental Procedures

Fungal Strains

Alternaria solani strain 584 [10], which produces solanapyrones, was used as a source of PKS gene cloning. *Aspergillus oryzae* M-2-3 was used as a host for fungal PKS expression [26].

Bacterial Strains and Plasmids

Escherichia coli XL1-Blue MRF and DH5α were from Stratagene (La Jolla, CA) and Clontech (Mountain View, CA), respectively. pT7-Blue T vector was from Novagen (Madison, WI). The cosmids pANsCos1 [39] and pOJ446 [24] were described previously.

Biochemicals, Chemicals, and Media

Unless specified otherwise, common biochemicals and chemicals were from standard commercial sources such as Sigma (St. Louis, MO) and Wako. *E. coli* strains carrying plasmids were grown in Luria-Bertani medium and were selected with appropriate antibiotics. Culture media for *A. solani* [14] and *A. oryzae* [40] were described previously. ^{13}C-labeled acetates and methonine (99% atom % ^{13}C) were obtained from ISOTEC (Miamisburg, OH).

A. solani Genomic Library Construction

A. solani genomic DNA prepared as previously described [40] was partially digested with Sau3AI, and fragments in the 30–40 kb size

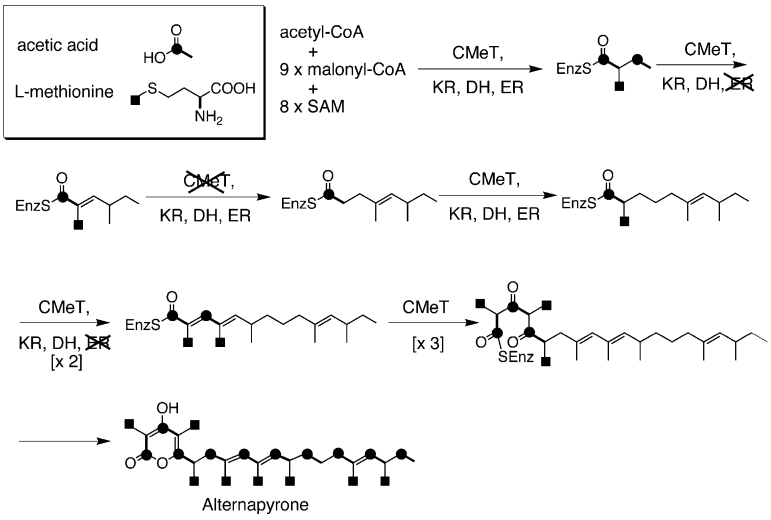


Figure 4. Proposed Biosynthesis of Alternapyrone Catalyzed by PKSN

range were cloned into cosmid vectors by in vitro packaging. The first genomic DNA library was constructed in the pANsCos1 cosmid vector [39] and the second genomic DNA library was constructed in the pOJ446 cosmid [24] as described in the literature.

PCR Amplification of *A. solani* β-Ketoacyl Synthase Domain

Amplification of the KS domain of *A. solani* was performed using the degenerate primers KSD-F (5′-GAR GCN CAY GGN ACN GGN AC-3′) and KSD-R (5′-CCA YTG NGC NCC YTG NCC-3′) designed from the conserved regions of KS domains of fungal RD-PKS genes. The approximately 900 bp DNA fragment amplified was gel purified from a 1% low-melting point agarose gel using a PCR Prep kit (Promega, Madison, WI) and subcloned into pT7-Blue T vector.

Library Screening

The *A. solani* genomic DNA library colonies (total 1 × 10^4) were transferred onto Nytran membranes (Schleicher & Schuell, Dassel, Germany) and fixed for hybridization following the instructions in the manufacturer's manual. The membranes were hybridized with the DIG-labeled fragment and positive signals were detected using a DIG Color Detection kit (Roche, Basel, Switzerland).

DNA Sequencing

Primer binding sites for sequencing were randomly inserted into the target plasmid using the GPS-1 Genome Priming System (New England Biolabs, Ipswich, MA), and resulting plasmids were sequenced by automated DNA sequencer models 4000L and 4200L (LI-COR,

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                                        I                                  II
A. solani PKSN          EYVGLIADKQPGLRILEICACTGGTTYHVLERLRNPDG-TSKAAQYYFTDISPGFLAKAADRFNQDASIMQFGTLNIENA   1523
A. terreus LDKS         ELVRLCCHKNPRARILEICGCTGGCTQLVVDSLGPNP----PVGRYDFTDVSAGFFEAARKRFAGWQNVMDFRKLDIEDD   1498
P. citrinum MlcB        ELIRLCAHKNPRSRILEICGCTGGCTKLIVNALGNTK----PIDRYDFTDVSAGFFESAREQFADWQDVMTFKKLDIESD   1531
A. terreus LNKS         ELVAQIAHRYQSMDILEICACTGGATKYVLATPQLG------FNSYTYTDISTGFFEQAREQFAPFEDRMVFEPLDIRRS   1501
P. citrinum MlcA        DLVSQIAHRYQSIDILEICLGTGIATKRVLASPQLG------FNSYTCTDISADVIGKAREQLSEFDGLMQFEALDINRS   1495
G. moniformis FUM1p     TAG----HTRPTLRVLEICACTGGGAQVILEGLTNGK--ERLFSTYAYTDISAGFFVAAQERFKAYKG-LDFKVLDITKD   1526
C. heteropstrophus PKS1 TYLHLLGHKKPSLRVLTVGPQSGPTSLNLLMLLAELGGGEIPFAVLHHSDAELNIDQTVRSRFPSWADSVGFRDVFNESG   1510
                           .          :* :*  :*  :  ::                 :* . ..   . . :    . : *  :

                                       III                                IV
A. solani PKSN          PTEQG--FSPELFDLIVCANVLHATKSIQETLTHCKLLLKPGGRLVLSDVTI-KRIFSGFIMGPLPGWWLGEADGRMGGP   1600
A. terreus LDKS         PEAQG--FVCGSYDVVLACQVLHATSNMQRTLTNVRKLLKPGGKLILVETTR-DELDLFFTFGLLPGWWLSEEPERQSTP   1575
P. citrinum MlcB        PEQQG--FECATYDVVVACQVLHATRCMKRTLSNVRKLLKPGGNLILVETTR-DQLDLFFTFGLLPGWWLSEEPERKSTP   1608
A. terreus LNKS         PAEQG--FEPHAYDLIIASNVLHATPDLEKTMAHARSLLKPGGQMVILEITHKEHTRLGFIFGLFADWWAGVDDGRCTEP   1579
P. citrinum MlcA        PAEQG--FKPHSYDLIIASDVLHASSNFEEKLAHIRSLLKPGGHLVTFGVTHREPARLAFISGLFADRWTGEDETRALSA   1573
G. moniformis FUM1p     PSEQG--FESGSFDLIIAGNVIHATPTLNETLANVRKLLAPEGYLFLQELSP-KMRMVNLIMGILPGWWLGAAEGRVEEP   1603
C. heteropstrophus PKS1 ASQQNPPIVNETYDIVVAFNVLGSSPGFSKTLSAAAPLLNARGKILLVDNSHKSPMAALVWGPLPSFLSTWVDEKSADSP   1590
                        .  *.  :    :*.::. :*: ::  :  .:     ** . * :.    :  .     .     .            .
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Figure 5. Amino Acid Sequence Alignment of *C*-Methyltransferase Domains in Fungal Iterative Type I Polyketide Synthases

An asterisk (*) marks completely conserved amino acid residues, whereas a colon (:) identifies highly conserved residues and a period (.) indicates moderately conserved residues. Numbers I, II, III, and IV correspond to consensus motifs I–IV following the definition of Anderson and coworkers [30]. Their putative key amino acids are shown in black.

Lincoln, NE) using a Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech, Piscataway, NJ). Sequencing with synthetic primers was also carried out using an ABI PRISM Genetic Analyzer with BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

Construction of PKSN Expression Plasmid and Transformation

To express PKSN, the full-length *alt5* gene was amplified by RED AccuTaq polymerase (Sigma) with primers *att*-PKSf (5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT ACT GCC ACC ATG GAC AAG CCA GTC-3′) and *att*-PKSr (5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT CAA CCT CAA GCC TGG CCA AGG-3′), which have *att*B1 and *att*B2 sequences at their 5′ ends, respectively, for GATEWAY cloning (Invitrogen). The amplified fragment was gel purified and submitted to BP reaction with donor vector pDONR 201. After transformation, four entry clones, pEntPKSN1–4, were obtained. These entry clones were then used in an LR reaction with pTAex3R destination vector which was converted from the expression vector pTAex3 [25]. The resulting expression plasmid pTA-PKSN was used to transform the fungal host *A. oryzae* M-2-3 by the protoplast-polyethylene glycol method previously described [26].

Thus obtained, ten transformants were grown in shake culture in Czapek-Dox medium with glucose as a carbon source for 6 days and then transferred into Czapek-Dox medium with starch to induce expression under the α-amylase promoter. Shake cultures were continued for a further 2 days. No specific product was found in the ethyl acetate extracts of the induction culture media of any of the transformants. However, production of a new compound was detected by TLC analysis of the mycelial acetone extracts of seven transformants. One of the transformants, designated *A. oryzae*/pTA-PKSN10a, was chosen for further analysis.

HPLC Analysis of Alternapyrone

The mycelial extract of induction culture of the transformant *A. oryzae*/pTA-PKSN10a was redissolved in CH_3CN and analyzed by HPLC on an ODS column (ODS-80Ts, 4.6 × 150 mm; Tosoh, Tokyo, Japan). Elution conditions were as follows: gradient system from 80% to 100% (v/v) CH_3CN in water over 10 min and then 100% (v/v) CH_3CN for 10 min; flow rate 0.8 ml/min at 40ºC. The PKSN product, alternapyrone, was eluted as a single main peak at Rt 13.3 min.

Analysis by Liquid Chromatography-Coupled Mass Spectrometry (LC-MS)

The *A. oryzae*/pTA-PKSN10a product was analyzed by an ion trap mass spectrometer (ThermoQuest LCQ, San Jose, CA) with atmospheric pressure chemical ionization (APCI) coupled to a TSK-Gel ODS-80Ts column (4.6 × 150 mm; Tosoh). HPLC conditions were the same as described above.

Feeding Experiments with ^{13}C-Labeled Acetates and Methionine

To a 200 ml induction culture of *A. oryzae*/pTA-PKSN10a in a 500 ml Erlenmeyer flask, 100 mg of sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate, sodium [1,2-$^{13}C_2$]acetate, and [methyl-^{13}C]methionine were added, separately, on day 2 of the growth of the induction culture. Four culture flasks were used for each feeding experiment. After 3 more days, labeled alternapyrone was isolated by silica gel column chromatography and analyzed by ^{13}C-NMR (α-500; JEOL, Tokyo, Japan).

Reverse Transcription-PCR

To confirm the presence of introns, total RNA was prepared from *A. oryzae*/pTA-PKSN10a mycelia after 1 day induction with starch. RT-PCR was carried out (Superscript One-Step RT-PCR System for Long Templates kit, Invitrogen) with eight pairs of primers to cover the entire PKSN cDNA. Eight cDNA fragments obtained were directly sequenced with the primers used to amplify the fragments.

Supplemental Data

Supplemental Data include NMR data of alternapyrone and can be found with this article online at http://www.chembiol.com/cgi/content/full/12/12/1301/DC1/.

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Accession Numbers

The nucleotide sequence reported here has been deposited in the DDBJ/GenBank/EMBL database under accession number AB120221.