

Novel Polyketide Synthase from *Nectria haematococca*

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We identified a polyketide synthase (PKS) gene, *pksN*, from a strain of *Nectria haematococca* by complementing a mutant unable to synthesize a red perithecial pigment. *pksN* encodes a 2,106-amino-acid polypeptide with conserved motifs characteristic of type I PKS enzymatic domains: β -ketoacyl synthase, acyltransferase, duplicated acyl carrier proteins, and thioesterase. The *pksN* product groups with the *Aspergillus nidulans* WA-type PKSs involved in conidial pigmentation and melanin, bikaverin, and aflatoxin biosynthetic pathways. Inactivation of *pksN* did not cause any visible change in fungal growth, asexual sporulation, or ascospore formation, suggesting that it is involved in a specific developmental function. We propose that *pksN* encodes a novel PKS required for the perithecial red pigment biosynthesis.

Polyketides comprise diverse natural products including antibiotics, pigments, and mycotoxins that are formed from small carbon precursor acids whose successive condensation is catalyzed by polyketide synthases (PKSs) (34). Filamentous fungi are prolific producers of polyketide metabolites of pharmacological and agricultural interest (4, 8, 13, 14, 28, 33, 42, 44, 46). These enzymes are quite diverse but belong to one of two basic types (23, 24). Modular PKS type I contains one or more large multifunctional protein subunits, while iterative PKS type II has only a few active sites on separate polypeptide chains. All identified fungal PKSs belong to modular type I, with a single polypeptide that contains up to eight domains for polyketide biosynthesis (21, 27). A typical fungal PKS consists of a linear succession of ketosynthetase (KS), acyltransferase (AT), dehydratase (DH), enoyl reductase (ER), ketoreductase (KR), acyl carrier protein (ACP), and thioesterase (TE) domains. In addition to the biochemically characterized enzymes, many other potential fungal PKSs have been identified by using degenerate-PCR approaches (5, 30, 36).

Over the past decade, several genes encoding fungal PKS enzymes involved in pigment production have been cloned and characterized. Most studies have focused on the biosynthesis of dark brown pigments, melanins (14, 35, 44), and green pigments (28, 33), because of their important role in fungal pathogenesis (29). Relatively little is known about the biosynthesis of red pigments, with the exception of the bikaverin produced by *Gibberella fujikuroi* (31). In *Nectria haematococca*, mutants in which the pigmentation of mycelia and perithecia is affected (i.e., mutants either show hyperproduction of red pigments or are albinos) have been obtained through mutagenesis (11, 40, 41). Extensive genetic analyses of such mutants have been conducted (11, 40). It has been established that at least six distinct loci control mycelial pigmentation. These pigmented metabolites are naphthoquinones (39), most of which have antibacterial, antifungal, insecticidal, and phytotoxic properties (26). The perithecial color phenotype appears to be governed

by at least two genetically independent loci, of which one is marked by the I_4 mutation (3). This mutation suppressed the red pigmentation of the perithecial wall but did not affect the color of the mycelium. This observation suggests that the I_4 gene affected a function that was expressed in the sexual phase but not in the vegetative phase. Perithecial color mutants have been reported to occur in several ascomycete species including *Hypomyces solani* (19), *Neurospora crassa* (22), *Magnaporthe grisea* (9), and *Gibberella fujikuroi* (7), but the genes involved in such variation have not been cloned. A red pigment also accumulates in the ascospores of *Aspergillus nidulans* (6). This pigment is an anthraquinone, which suggests that it is a product of a PKS that has not yet been characterized.

Our objective in this study was to clone a gene involved in the biosynthesis of a perithecial red pigment in *Nectria haematococca*. Such genes might also be conserved in other fungi that produce red pigments during the sexual phase or play a role in the biosynthesis of other secondary metabolites.

MATERIALS AND METHODS

Fungal strains, media, and crosses. All strains are derived from a homothallic species within the *N. haematococca* complex CBS 225.58 (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands). Based on DNA sequences of the 28S ribosomal DNA published in reference 17, we assigned this strain to clade 3 of the *Fusarium solani* complex established by O'Donnell (37) (data not shown).

A mutant strain ($a_1 s_1 I_4 mod$) (hereafter referred to as [$a_1 s_1 I_4 mod$]) was used as recipient in transformation experiments (16). The a_1 and s_1 mutations prevent the appearance of two morphological modifications called the "Anneau" and the "Secteur," respectively (16, 43). The I_4 mutant produces white, rather than red, perithecia and was obtained by treatment with *N*-methyl-*N'*-nitro-nitrosoguanidine (3). The *mod* mutation prevents ascospore formation within perithecia. The I_4 and *mod* mutations are genetically linked (0.5% of recombination frequency) (3). A strain containing the a^*_{58} mutation (hereafter referred to as [a^*_{58}]), which has altered "Anneau" expression (10), produced red perithecia and was used in some crosses.

General culture conditions and manipulations were as previously described (10). Strains were purified via subculture of microconidia and maintained at 26°C on potato dextrose agar made of potato broth (potatoes [200 g/liter] boiled for 1 h, peeled, and sliced) and glucose (20 g/liter) supplemented with agar (20 g/liter). Long-term stocks were stored as agar plugs under mineral oil at 12°C.

Crossing methods for the homothallic species *Fusarium* sp. strain CBS 225.58 have been described elsewhere (3, 40). When PR1, a transformant with a wild-type phenotype, was crossed with [a^*_{58}], which produces red fertile perithecia, a preliminary analysis of a sample of descendants from each perithecium collected on the line of contact was required to distinguish hybrids from parental perithecia (11). The progeny from two hybrid perithecia (250 ascospores from each

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perithecium) were analyzed for color marker segregation (3, 40), although these progeny may not all have originated from independent meiotic events.

DNA and RNA manipulation. All nucleic acid manipulations were performed by standard methods (2). For Southern blot analysis, genomic DNA was extracted as described previously (32). The nucleotide sequence of the mutant allele I_4 was determined from two independent amplicons obtained following PCR with oligonucleotide primers I4-187 (5'-GGCGCTTGAATGCTTGATA G-3') and ip2393c (5'-TGGCCATCTTCTCGAATCGT-3') by using the following program: 30 cycles of 1 min at 96°C, 30 s at 56°C, and 3 min at 72°C, followed by a final extension of 5 min at 72°C.

Transformation of *N. haematococca*. Transformation was performed as previously described (16), with hygromycin B resistance as the selectable marker. For transformation with cosmids carrying the hygromycin resistance gene (*hph*), 10 µg of each cosmid pool was used. When cotransformation experiments were performed, usually with plasmid pBC-Hygro, 5 µg of the transforming plasmid was mixed with 5 µg of the DNA to be tested. Transformants were selected on PH8 medium (potato dextrose agar with 200 g of sucrose/liter, 3 g of agar/liter, and 8 mg of hygromycin B/liter) (Sigma-Aldrich).

Cosmid library screening. We constructed an "XSG" gene library (16) by inserting genomic DNA from the *N. haematococca* wild-type strain partially digested with XhoI into the pMOCosX cosmid as previously described (38). We tested 20 cosmid pools in transformation experiments with [*a₁S₁I₄mod*] as the recipient. An average of 400 hygromycin-resistant transformants per cosmid pool were cultured on PH8. After 7 days of growth, plates were exposed to daylight, and 15 days later they were examined for perithecial color. One cosmid pool, XSG11, led to the production of red perithecia. The complementing cosmid was identified by examining 20 subpools corresponding to each of the 12 columns and eight rows of the microtiter plate.

Subcloning of cosmid E12. E12 was digested with BamHI, ClaI, EcoRI, HindIII, PstI, SalI, and XhoI. Pools of fragments larger than 1 kb were eluted from the agarose gel and used to cotransform the [*a₁S₁I₄mod*] strain. Fragments of pools restoring the I_4^+ phenotype were cloned into pBluescript and then tested in the same way. pI4C3-2 was derived from E12 and carried a 4.5-kb ClaI fragment containing a truncated *pksN* gene.

Sequencing. The insert in pI4C3-2 was entirely sequenced on both strands by using synthetic primers and the ABI PRISM Dye-terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif.). The ends of each cloned ClaI fragment from cosmid E12 (pI4C3-1 to -5) were sequenced with plasmid-specific primers on an ABI373 automated DNA sequencer (Applied Biosystems).

Phylogenetic analysis. Phylograms were inferred from concatenated ClustalX (41) protein multiple alignments of the AT and KS conserved domains. Neighbor-joining analyses were performed with Phylo_win (15) with the PAM (Point Accepted Mutation) distance matrix. One thousand bootstrap replicates were performed.

Nucleotide sequence accession numbers. The nucleotide sequence of *pksN* was deposited in the GenBank and EMBL databases under accession number AY487573. The domain alignments and phylogenetic trees were deposited in the tree base database under accession number 26358.

RESULTS

Cloning of the I_4^+ gene by sib selection. One cosmid pool, XSG11, transformed strain [*a₁S₁I₄mod*] so that it produced red perithecia at a low frequency (3 of 250 transformants recovered). One transformant (PR1) had a wild-type phenotype, and two other transformants produced a mixture of red and white perithecia, suggesting instability of the transforming DNA. When the stable PR1 transformant was crossed with [*a*₅₈*], which carries the I_4^+ allele, all of the progeny produced red perithecia. Thus, the complementing cosmid probably integrated at I_4 , suggesting that this cosmid carries the I_4^+ allele rather than a suppressor of the I_4 mutation. In bidimensional subpooling experiments, only subpools E and 12 yielded transformants with red perithecia, thus identifying E12 as the complementing cosmid. When this cosmid was used to transform [*a₁S₁I₄mod*], two types of transformants were recovered. A few (14%) transformants formed only red perithecia (stable transformants), while the remainder (86%) formed a mixture of red

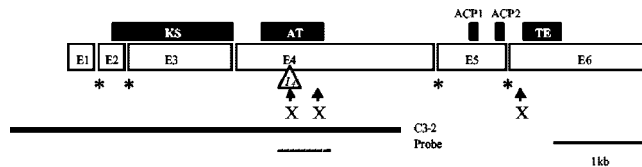


FIG. 1. Diagram of the *pksN* gene showing six exons (E1 to E6) and five introns (intervals between E1 to E6). Conserved positions of introns relative to the *wA* gene are indicated by asterisks. Conserved amino acids at catalytic sites are indicated by boxes whose sizes correspond to the aligned domains KS, AT, ACP, and thioesterase (TE). The solid line indicates the length of the C3-2 fragment used in transformation experiments. The 0.6-kb PCR fragment (dashed line) was used as a probe in Southern blot experiments. X, XbaI site (the first one is absent in the I_4 mutant).

and white perithecia (unstable transformants), even when purified by subculturing single uninucleate conidia. pI4C3-2, a subclone of E12 carrying a 4.5-kb ClaI fragment, transformed strain [*a₁S₁I₄mod*] so that it produced red perithecia at low frequency.

Identification and analysis of a polyketide synthase gene. Analysis of the 4.5-kb fragment with the blastx algorithm (1) identified an open reading frame of 1,214 residues with a high degree of similarity to several type I PKS enzymes. The highest similarity was to the PKS encoded by the *wA* gene from *A. nidulans* (33). The 4.5-kb DNA fragment contains a 3'-truncated gene that we designated *pksN*. The complete *pksN* gene sequence was obtained from cosmid E12. Based on protein alignments and the presence of nucleotide sequences that closely matched the 5' and 3' end splice and internal consensus sequences (18), five putative introns ranging in size from 45 to 54 nucleotides were inferred (Fig. 1).

The predicted 2,106-amino-acid translation product of *pksN* contains five conserved functional domains (Fig. 1): a β -ketoacyl synthase domain (KS), an acyltransferase domain (AT), two acyl carrier protein motifs (ACP1 and ACP2), and a thioesterase domain (TE). Based on the organization of the functional domains, PKSN is of the WA type, which includes PKSs involved in pigment biosynthesis (14, 28, 33, 44) and in the aflatoxin/sterigmatocystin biosynthetic pathways (8, 13, 47). Phylogenetic analyses based on the KS (440 amino acids) and AT (240 amino acids) domains revealed similar evolutionary relationships. As shown on the tree obtained by combining domains AT and KS (Fig. 2), bootstrap values support the grouping of PKNS within the WA-type group.

PKSN and perithecium pigmentation. The I_4 allele results from an 8-bp deletion at position 2422 downstream of the ATG codon. This mutation results in a stop codon 20 bp downstream of the mutation and thus encodes a truncated protein of 763 residues containing only the β -ketosynthase domain. To confirm that the I_4 phenotype was not due to a secondary mutation, we cotransformed this strain with the pBC-Hygro plasmid and the I4C3-2 fragment, which lacks a portion of the 3' region of *pksN* (Fig. 1). In the resulting analysis, some transformants were restored to the production of red perithecia and were presumed to result from an integration of the plasmid into the I_4 locus. This hypothesis was tested by using a PCR approach based on the loss of an XbaI site associated with the deletion

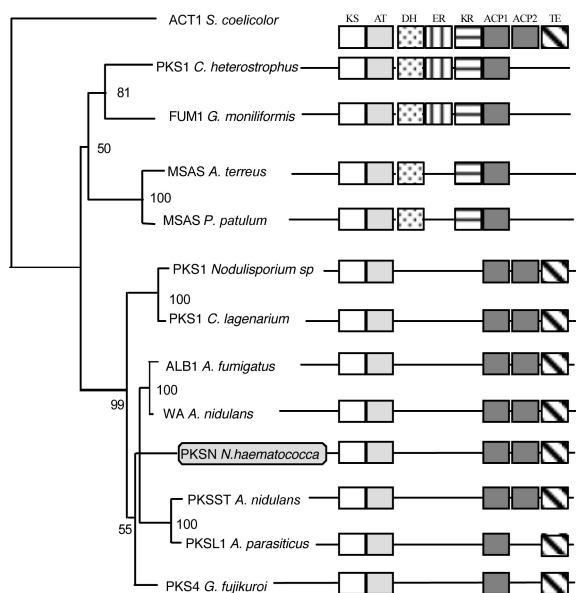


FIG. 2. Phylogenetic relationships of fungal PKSs inferred from the combined KS and AT domains. Corresponding organizations of the different functional domains within each fungal PKS are presented to the right. Domains are described in the top boxes, with the same abbreviations as for WA-type PKSs. Other domains are labeled as follows: dehydratase (DH), enoyl reductase (ER), and β -ketoacyl reductase (KR). Organisms and accession numbers (in parentheses): *A. fumigatus* ALB1 (X65866); *A. nidulans* wA (X65866); *G. fujikuroi* PKS4 (AF278141); *Aspergillus parasiticus* PKSL1 (L42765); *N. haematococca* PKS_N (AY487573); *Nodulisporium* sp. PKS1 (AF151553); *Colletotrichum lagenarium* PKS1 (D83643); *A. nidulans* PKSST (L39121); *Streptomyces coelicolor* ACT1 (Q02059); *C. heterostrophus* PKS1 (U68040); *G. moniliformis* FUM5 (AF155773); *Aspergillus terreus* MSAS (U31329); *Pittosporum patulum* MSAS (X55776).

in the I_4 allele. After amplification of a 1.9-kb fragment spanning the deletion and subsequent digestion with XbaI, we observed cleavage of the fragment in two C3-2 transformants with red perithecia and in the wild type but not in the [$a_1s_1I_4mod$] strain (data not shown). The same two transformants were analyzed by Southern blotting (Fig. 3). The mutant lost a 0.4-kb band, and a 7.1-kb band was shifted to 7.5 kb. The two transformants had the same wild-type profile at the I_4 locus, demonstrating that the red perithecial phenotype results from gene replacement. The additional hybridizing band in one transformant presumably results from an ectopic integration of another copy of the C3-2 fragment.

Location of putative regulatory sites in the promoter of *pksN*. The promoter region of *pksN* was examined for the presence of putative binding sites for fungal transcription factors. In *pksN*, we found two binding sites for AflR, the binuclear zinc cluster transcription factor required for gene expression in both the sterigmatocystin and aflatoxin pathways, three binding sites for AreA, a factor regulating genes involved in nitrogen metabolism, and three binding sites for PacC, which regulates genes based on ambient pH signals (Table 1). The AflR sites found in *pksN* are at the same distance (120 bp) from the translational initiation codon as those found for *pksA*, involved in aflatoxin biosynthesis in *Aspergillus* (12).

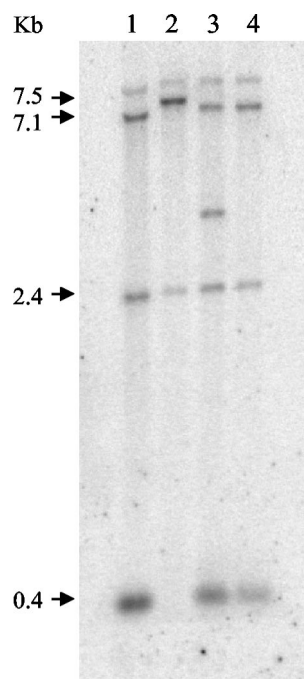


FIG. 3. Southern blot analysis of strains transformed with the C3-2 linear fragment. Genomic DNAs were digested by XbaI, electrophoresed on a 0.8% agarose gel, transferred to a nitrocellulose membrane, and hybridized with a ³²P-labeled 663-bp PCR-amplified fragment of *pksN* spanning the two XbaI sites surrounding the I_4 mutation (Fig. 1). Lane 1, [a^*_{s8}] (I_4^+ allele); lane 2, [$a_1s_1I_4mod$] (mutated allele); lanes 3 and 4, transformants E11 and E53, respectively, with restored I_4^+ perithecial red pigmentation.

DISCUSSION

We isolated a type I PKS gene (*pksN*) that participates in the biosynthesis of the red pigment present in the perithecial wall of *N. haematococca*. Prior to this study, the biosynthetic origin of the pigment in the perithecial wall was unknown. The demonstration that I_4 encodes a PKS is consistent with the hypothesis that this red perithecial pigment also is a polyketide. Inactivation of the gene did not lead to significant changes with respect to hyphal growth, asexual spore formation, time course of perithecial formation, or sexual spore production. To our knowledge, this is the first report of the isolation and characterization of a fungal gene involved in the biosynthesis of a red pigment present in a specific cell type of the sexual stage. Although the precise function of this pigment has not yet been established, on the basis of what is known for other fungal spore pigments, particularly melanins (29), it probably protects the ascospores from environmental stresses such as UV irradiation and desiccation. It also could have an inhibitory effect

TABLE 1. Locations of putative regulatory sites in the promoter of *pksN*

Fungal transcription factor	Locations of binding sites (bp) ^a
AflR.....	-310, -143
AreA.....	-599, -569, -354
PacC.....	-161, -116, -96

^a Distance from the translational initiation codon.

on ascospore germination, since germination was never observed to occur inside red perithecia but does occur within the uncolored perithecia.

This novel PKS provides an opportunity to investigate the biosynthesis of red pigments accumulating in a specific cell type, e.g., ascospores or cleistothecia, in other fungi and the relationship between spore pigments and other secondary metabolites. The presence in the promoter of *pksN* of putative regulatory sites for three transcription factors, the aflatoxin pathway-specific regulatory protein, AflR, and the global regulatory proteins, AreA and PacC, also found in the promoter of *pksA*, involved in aflatoxin biosynthesis (12), is very intriguing. This finding suggests that the expressions of *pksN* and *pksA* are influenced by the same factors. In addition, the fact that the red pigment of *A. nidulans* ascospores is structurally similar to norsoloric acid, the first stable intermediate in the biosynthesis of aflatoxins (6), raises interesting questions about an evolutionary relationship between spore pigments and mycotoxins.

In addition, the results reported here show that phylogenetic analysis of revised alignments of fungal PKSs clarified the relationship between them. For example, the phylogenetic position of PKS1 from *C. heterostrophus* (45), which produces the T toxin, was not clearly established on the basis of the alignment of 240 amino acids of the KS domain (5). When the analyzed region is increased to 440 residues and the *FUMI* gene product from *G. moniliformis* is included, both of these genes cluster with the 6-methylsalicylic synthase (MSAS)-type PKSs. Clustering with either AT, KS, or both domains appears to be correlated with the presence of the dehydratase and ketoreductase domains. Increasing the size of the aligned AT region from 50 to 240 amino acids also identified more highly conserved sequence regions. Therefore the AT and KS domains could be used together to develop degenerate-PCR approaches to efficiently screen for PKS genes.

By sequencing the genomic region surrounding the *pksN* gene, we also detected other open reading frames with predicted P-450 monooxygenase and *O*-methyl transferase activities. These enzymatic activities are associated with a PKS in several mycotoxin-biosynthetic pathways (20, 25) and could also be involved in the biosynthesis of the red pigment. Inactivation experiments are in progress in order to determine if these genes are coregulated and part of a new gene cluster.

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