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Characterization of two polyketide synthase genes in *Exophiala lecanii-corni*, a melanized fungus with bioremediation potential

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Abstract

Exophiala lecanii-corni has significant bioremediation potential because it can degrade a wide range of volatile organic compounds. In order to identify sites for the insertion of genes that might enhance this potential, a genetic analysis of *E. lecanii-corni* was undertaken. Two polyketide synthase genes, *ElPKS1* and *ElPKS2*, have now been discovered by a PCR-based strategy. *ElPKS1* was isolated by a marker rescue technique. The nucleotide sequence of *ElPKS1* consists of a 6576-bp open reading frame encoding a protein with 2192 amino acids, which was interrupted by a 60-bp intron near the 5′ end and a 54-bp intron near the 3′ end. Sequence analysis, results from disruption experiments, and physiological tests showed that *ElPKS1* encoded a polyketide synthase required for melanin biosynthesis. Since *ElPKS1* is non-essential, it is a desirable bioengineering target site for the insertion of native and foreign genes. The successful expression of these genes could enhance the bioremediation capability of the organism. *ElPKS2* was cloned by colony hybridization screening of a partial genomic library with an *ElPKS2* PCR product. *ElPKS2* had a 6465-bp open reading frame that encoded 2155 amino acids and had introns of 56, 67, 54, and 71 bp. Although sequence analysis of the derived protein of *ElPKS2* confirmed the polyketide synthase nature of its protein product, the function of that product remains unclear.

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1. Introduction

Large quantities of volatile organic compounds (VOCs) are generated each year in industrial processes and result in considerable environmental contamination [1]. The United States Clean Air Act Amendments of 1990 mandated stricter regulations for VOCs, increasing the demand for effective abatement technologies. Air pollution control with bioremediation is attractive due to its low operating cost and ease of use [2]. Vapor-phase bioreactors containing fungal biofilms can potentially remove more VOCs than bacterial biofilms from off-gas streams under even stricter conditions. For example, *Exophiala jeanselmei*, a black, dimorphic, and zoopathogenic fungus, has been used extensively in fungal bioreactor studies with considerable success [3]. An environmental isolate of *Exophiala lecanii-corni*, a species closely related to *E. jeanselmei* that is similarly highly melanized and dimorphic but not known to be a human pathogen, also has significant potential as a bioreactor agent, because it can degrade many VOCs under a variety of conditions [4,5]. These attributes, coupled with its lack of pathogenicity, make *E. lecanii-corni* a particularly appealing organism for use in bioreactors.

Polyketide metabolites are structurally diverse natural products with a variety of biological activities that are used as antibiotics, immunosuppressants, antiparasitics, and insecticides [6–8]. Polyketide metabolites are also common components of melanins, which are dark pigments composed of various types of phenolic or indolic monomers. Melanins are often complexed with protein and less often with carbohydrates. These secondary metabolites are not essential for growth and development, but probably enhance the survival and competitive abilities of fungi in certain environments [9–11]. Several types of melanin are found in fungi, such as dihydroxyphenylalanine (DOPA) melanin, catechol-melanin, γ-glutaminyl-3,4-dihydroxybenzene (GDHB) melanin, and 1,8-dihydroxynaphthalene (DHN) melanin, and are named according to their composition and method of synthesis [11]. Among them, the best-characterized fungal melanin is DHN melanin [10,12]. DHN is synthesized from one acetyl-CoA molecule and four malonyl-CoA molecules, through a head-to-tail joining and cyclization catalyzed by a type-I polyketide synthase (Pksp), to form 1,3,6,8-tetrahydroxynaphthalene (THN). Subsequent enzyme-catalyzed steps produce the 1,8-DHN, which is then polymerized to melanin [10,12,13].

A type-I Pksp is a large multifunctional protein that usually has four conserved domains: a β-ketoacyl synthase domain, an acyl transferase domain, one or two acyl carrier domains, and a thioesterase domain [12,14]. Disruption of the gene (*WdPKS1*), which encodes a type-I Pksp involved in melanin biosynthesis in *Wangiella (Exophiala) dermatitidis*, a human pathogen closely related to *E. lecanii-corni* and *E. jeanselmei*, produces albino strains [15,16]. We also found that native and foreign genes and mutant alleles can be successfully transferred into the non-essential *WdPKS* locus, and expressed and overexpressed for functional comparisons of their

products, without adversely affecting the characteristics of *W. dermatitidis*, except for reducing its pathogenicity [16–18]. These observations suggested that the melanin polyketide synthase gene of *E. lecanii-corni* (*ElPKS*) might be a target into which key genes involved in VOC degradation could be integrated and expressed. The resulting bioengineered fungus might then be more efficient in degrading an even wider variety of VOCs than its parent. In the course of this work, two different *ElPKS* genes were discovered in *E. lecanii-corni*. The purpose of this report is to describe these two genes, confirm that *ElPKS1* is required for melanin biosynthesis, and describe our finding of a second *ElPKS* gene in this fungus.

2. Experimental procedures

2.1. Strains and culture conditions

Exophiala lecanii-corni CBS 102400 served as the parent of all the strains derived in this study [4,5]. *Escherichia coli* XL-Blue (New England Biolabs, Beverly, MA) was used as the host strain for propagating the vectors, pBluescript II KS (+/−) (Stratagene, La Jolla, CA) was used for the construction of the *E. lecanii-corni* genomic library and for deriving subclones for sequencing, and pGEM-T Easy (Promega, Madison, WI) was the vector used for propagating PCR products. pCB1004 (kindly provided by J. Sweigard, DuPont, Wilmington, DE), a pBC SK (+/−)-derived plasmid containing an *E. coli* hygromycin phosphotransferase gene (*hph*), which is a dominant marker for selecting hygromycin B^- (HmB^-, Sigma, St. Louis, MO) resistant *E. lecanii-corni* transformants, was used for the construction of all the *ElPKS* disruption vectors [19]. Cultures of *E. lecanii-corni* were grown at 25 °C in yeast extract, peptone, dextrose (YPD) broth and a basal medium (BM),[1] as described previously [5,20]. When selection of transformants was required, these media were supplemented with HmB (50 μg/mL). Cultures of *E. coli* were grown at 37 °C in Luria–Bertani (LB) medium [20], which was supplemented as necessary for selection of transformants with ampicillin (100 μg/mL) or chloramphenicol (25 μg/mL) resistance.

2.2. Preparation and analysis of nucleic acid

Exophiala lecanii-corni genomic DNA was isolated by detergent lysis, phenol–chloroform extraction, and ethanol precipitation as previously described for *W. dermatitidis* [15]. *E. lecanii-corni* total RNA was isolated by a hot acidic phenol method

[1] *Abbreviations used:* BM, basal medium; dNTP, deoxynucleotide triphosphate; DHN, 1,8-dihydroxynapthalene; DOPA, dihydroxyphenylalanine; *ElPKS*, *E. lecanii-corni* polyketide synthase gene; G, glucose; GDHB, γ-glutaminyl-3,4-dihydroxybenzene; HBA, 4-hydroxybenzoic acid; HmB, hygromycin B; *hph*, hygromycin phosphotransferase gene; LB, Luria–Bertani; ORF, open reading frame; Pksp, polyketide synthase, PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; UTS, untranslated sequence; TLC, thin-layer chromatography; THN, 1,3,6,8-tetrahydroxynaphthalene; VOC, volatile organic compound; YPD, yeast peptone dextrose agar.

and treated with DNase [20]. Southern blotting experiments were performed by using standard methods [20]. PCRs were carried out with a Perkin–Elmer thermal cycler (Norwalk, CT). PCR mixtures usually contained 30 pmol of each primer, 100 ng of sample DNA, and deoxynucleotide triphosphates (dNTPs) at a final concentration of 200 μM each, 10× reaction buffer, 2 mM $MgCl_2$, and 4 U of *Taq* DNA polymerase (Promega). The conditions used for the PCR were: initial denaturation at 94 °C, 2 min, 36 cycles of denaturation (94 °C, 40 s), annealing (58 °C, 1 min), and extension (72 °C, 1 min) and a final extension at 72 °C, 8 min. Sequencing of the *ElPKS* gene fragments was carried out by The University of Texas Institute for Cellular and Molecular Biology Core Facility (Austin, TX). Sequence analysis was carried out using the NCBI BLAST and EMBI-EBI CLUSTAL-W tools available at the EMBL Web site (http://www.ebi.ac.uk/clustalw/index.html).

2.3. Transformation of E. lecanii-corni

Freshly cultured yeast cells were used to inoculate YPD (300 mL/1 L flask) broth at a concentration of 10^5 cells/mL. After 36 h at 25 °C with shaking, cells were harvested at a concentration of 10^6 cells/mL, filtered through a cotton layer to remove hyphae, transferred to a 250-mL centrifuge tube, chilled on ice for 30 min, and then collected by centrifugation (4000*g* for 8 min). The resulting cell pellet was resuspended, washed twice with ice-cold water, and then resuspended in ice-cold water to a final concentration of 10^9 cells/mL. An aliquot (90 μL) of the resulting competent cells was combined with 1–5 μg of linearized vector DNA and incubated on ice for 10 min. The competent cells were then subjected to electroporation in a Genepulser electroporation system (Bio-Rad, Richmond, CA) in a 0.2 mm cuvette at 1.5 kV field strength, 200 Ω resistance, and 25 mF capacitance, corresponding to a constant time interval within the range 4–6 ms. The electroporated cells were incubated in YPD broth (1 mL) with shaking at 25 °C for 2–4 h, and spread on YPD agar plates containing HmB and incubated at 25 °C until transformant colonies became evident.

2.4. Marker rescue of ElPKS1 and construction and screening of a partial genomic DNA library for the isolation of ElPKS2

The disruption vector pCQ700, linearized with *Bsa*I, was constructed by incorporating a 779-bp PCR fragment of *ElPKS1* into pCB1004 at its multiple cloning site. Transformants were selected based on their HmB resistance, and strains with the vector putatively inserted into the *ElPKS1* locus were identified by their albino phenotype. A marker rescue approach [15] was used to isolate the full length *ElPKS1* gene of *E. lecanii-corni*. DNA from the albino strains was isolated and digested with *Sac*II and *Xho*I, and the resulting DNA fragments were then treated by phenol–chloroform extraction, followed by ethanol precipitation. The purified fragments were allowed to self-ligate to produce the rescue plasmids pCQ58 and pCQ51, which were introduced into *E. coli* XL-Blue competent cells by electroporation. Colonies with putative clones of *ElPKS1* were selected on LB agar using chloramphenicol. For

the isolation of *ElPKS2*, *E. lecanii-corni* genomic DNA was digested with *Sac*I, *Sac*II, and *Xba*I to generate Southern blotting samples and probed with the *ElPKS2* 592-bp PCR product to identify the size region of DNA fragments that contained *ElPKS2*. Appropriately sized DNA fragments were collected from an agarose gel by use of a Gel Extraction Kit (Qiagen, Valencia, CA). pBluescript vector, digested with the same restriction enzymes as the fragments and dephosphorylated, was then ligated with the fragments. Ligation products (1–5 μg DNA) were introduced into *E. coli* Xl-Blue competent cells by electroporation. Transformed cells were spread on LB agar medium that contained ampicillin, and the resulting library was subjected to screening with the *ElPKS2* 592-bp PCR product as a probe.

2.5. PCR and RT-PCR to confirm intron sequences in ElPKS1 and ElPKS2

Several primer sets (Table 1) were designed based on BLAST analysis of the deduced amino acids sequences of *ElPKS1* and *ElPKS2*. *E. lecanii-corni* total RNA was isolated using a hot acidic phenol method [20], treated at 37 °C with RQ-DNase (Promega) for 1 h, followed by phenol–chloroform extraction, ethanol precipitation and washing. After the RNA was dissolved in RNase free water, RT-PCR was carried out using the Access RT-PCR system (Promega) in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The RT-PCR (total volume of 25 μL) consisted of 2.5 μL buffer (10×), 1 μL $MgSO_4$ (25 mM), 0.5 μL dNTPs (10 mM), 0.5 μL *Avian myeloblastosis* virus RT, 0.5 μL *Tfl* DNA polymerase, 5 μL total RNA (100 ng), 1 μL forward and reverse primer each (30 pmol), and a sufficient quantity of water to make a final volume of 25 μL. The thermal cycling conditions used were: 48 °C, 1 h; 94 °C, 2 min; followed by 36 cycles of 94 °C, 0.5 min, 58 °C, 1 min, and 68 °C, 1 min; and a final extension step at 68 °C for 6 min. The RT-PCR products were eluted from agarose gels, ligated with pGEM-T Easy (Promega) vector, and sequenced. The nucleotide sequences of the *ElPKS1* and *ElPKS2* genes were assigned GenBank Accession Nos. AY149213 and AY150178, respectively.

2.6. Thin-layer chromatography (TLC)

Exophiala lecanii-corni was grown on YPD agar plates until the surface was covered by the fungus. Cells were harvested by scrapping with a blade. Approximately a 10-mL packed cell volume was added to chloroform (20 mL) in a 50-mL plastic tube for overnight extraction with shaking at room temperature. After the extracts were separated from the cells by centrifugation, the chloroform portion was concentrated by evaporation. Samples were then loaded several times at the same spot on thin-layer chromatography plates (Whatman, Silica gel 150A K5F; Maidenstone, England), and finally developed with a mixture of chloroform: ethyl acetate (95:5) [21]. Extracted products were examined and photographed with a Fotoprep-I UV Transilluminator and Camera System (Cambridge, MA) and Polaroid Type 667 instant film under UV radiation at 300 nm.

Table 1
Primers, products, and gene positions related to the introns of *ElPKS2* and *ElPKS2*

Primer	Sequence	PCR (bp)	RT-PCR (bp)	Intron (bp)	Position (bp)[a]
EIPKS1					
Pk1f1	CAGAATCGAGAACAGAAACCG	312	252	60	1294–1354
Pk2r1	CTGTCAATGAGTCTGCAGAGG				
Pk1f2	GCTGATGACCCGAAGAGCATG	326	272	54	7574–7628
Pk1r2	CTCAGGAAGCAAGCAAGTCGT				
EIPKS2					
Pk2f1	GATGGCTCGCTTCCTTTGCACCCG	344	288	56	934–999
Pk2r1	CTCTTCTGGGTGGCTTTCGTACCA				
Pk2f2	CGACCACGATTAGAGACGAG	554	498	57	1282–2339
Pk2r2	CTCGCTTGGTAGTATTCACCT				
Pk2f3	TTGCGATAGTAGGAATGGCTG	337	283	54	1999–2053
Pk2r3	AGCTGTGACCAGAGCCAAGCG				
Pk2f4	CGCTTGGCTCTGGTCACAGCT	350	279	71	2408–2479
Pk2r4	GGCTACATTGAGCGCTACTGC				

[a] Nucleotides in the gene sequence encompassed by the intron.

2.7. Disruption mutant characterization

Cellular morphology was determined by using a Zeiss ICM 405 inverted microscope (Carl Zeiss, Oberkochen, Germany). Growth data were collected by counting cells with a hemacytometer and a Zeiss Standard WL microscope (Carl Zeiss). Feeding experiments with scytalone, kindly provided by M.H. Wheeler, National Cotton Pathology Research Laboratory, College Station, Texas, were carried out as described previously [15].

3. Results

3.1. PCR amplification two different ELPKS gene fragments

To amplify a portion of an *ElPK*S gene of *E. lecanii-corni*, two sets of degenerate primers were designed on the basis of conserved regions of Pksp known to be involved in melanin biosynthesis in other fungi. The sequences of these primers were as follows: Pelpksf1: 5′-GACCYKGCCTGWTCMWCYA-3′; Pelpksr1: 5′-GCKGC ACTRAAGTTGTTGA-3′; Pelpksf2: 5′-GCACGRTCRTCMTGAAGCGYCT-3′; Pelpksr2: 5′-GAAGGCAATATKGACRTTGCGC-3′, with Y being either C or T, K either T or G, W either A or T, M either A or C, and R either A or G. Two products were amplified from the genomic DNA of *E. lecanii-corni* with sizes of 779 and 592 bp. The two PCR products represented portions of two different *PKS* genes, which were designated *ElPKS1* and *ElPKS2*. A comparison of their deduced amino acid sequences confirmed these genes were homologs and represented amplicons of the same gene region, which encoded amino acids 607–782 of the β-ketoacyl synthase domain of the type-I Pksp of *W. dermatitidis* [15].

3.2. Cloning and characterization of ElPKS1

Transformation of *E. lecanii-corni* with the disruption vector pCQ700 (Fig. 1A), constructed by ligation of the 779-bp PCR product into pCB1004 and linearized by *Bsa*I, produced numerous HmB-resistant transformants (Fig. 2A). Among these, seven were albino suggesting that a gene (*ElPKS1*) involved in melanin biosynthesis had been disrupted. At least one albino (*elpks1*Δ-*A*) strain had a site-specific vector insertion in the *ElPKS1* gene (Fig. 1B), whereas all black transformants had ectopic plasmid integrations (data not shown). In feeding experiments with scytalone, a DHN melanin pathway intermediate, the albino strains were returned back to a wild-type-like phenotype (Fig. 2B).

The disruption of *ElPKS1* allowed its cloning by a marker rescue approach (Fig. 1A). Plasmid pCQ58, which carried the 5′-end portion of *ElPKS1*, was recovered by digesting genomic DNA isolated from *elpks1*Δ-*A1* with *Sac*II, whereas the 3′-end portion of *ElPKS1* was recovered in plasmid pCQ51 after incomplete *Xho*I digestion of the mutant genomic DNA. Following restriction mapping and a series of subcloning experiments, *ElPKS1* was completely sequenced. A total of 8641 bp

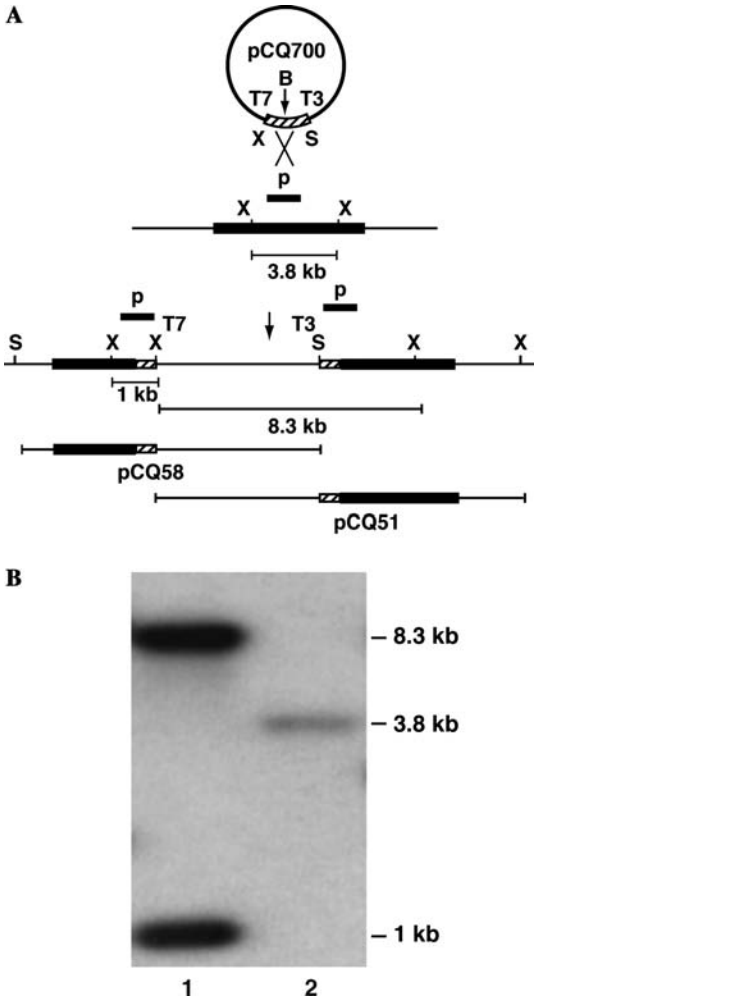

Fig. 1. *ElPKS1* disruption and marker rescue strategies and Southern blotting analysis. (A) Disruption plasmid pCQ700 was constructed by ligation of a 700-bp PCR fragment (hatched box) of *ElPKS1* into pCB1004, which was linearized with *Bsa*I (designated B) before transformation of *E. lecanii-corni*. The DNA isolated from the albino transformant was treated with *Sac*II (S) and *Xho*I (X) separately, and then the resulting fragments were purified and allowed to self-ligate prior to *E. coli* transformation. The 5′ portion of *ElPKS1* was recovered in pCQ58 and 3′ portion in pCQ51. (B) Southern analysis of an albino *elpks1*Δ-*A1*) transformant with the *ElPKS1* 0.7-kb PCR fragment for the probe (p). When digested by *Xho*I, the DNA fragments of *ElPKS1* from *ekpks1*Δ-*A1* were 8.3 and 1 kb as expected (lane 1), whereas that of the wild type was 3.8 kb (lane 2).

was sequenced, which included a considerable amount of upstream untranslated sequence (UTS), the *ElPKS1* ORF of 6576 bp that encoded 2192 amino acids, two introns and the termination sequence. The positions and sizes of the two introns were confirmed by RT-PCR and by sequencing the RT-PCR products (Table 1).

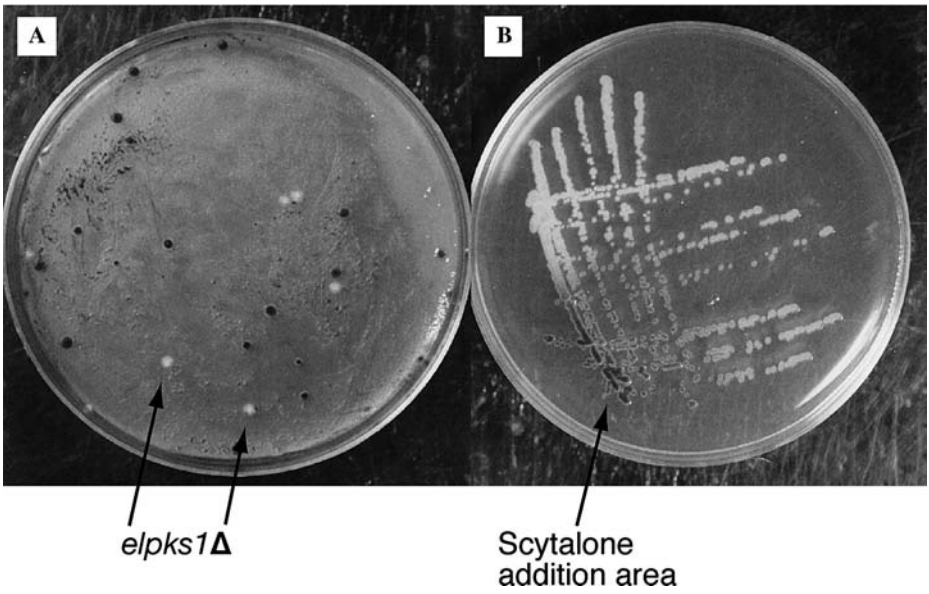

Fig. 2. Identification and selection of *elpks1*Δ-*A* mutants. (A) Putative *elpks1*Δ strains resulting from the integration of pCQ700 specifically into the *ElPKS1* locus were initially selected by resistance to HmB and by their albino phenotype (indicated by arrows). Transformation cultures were incubated at 25 °C for 8 days, prior to photography. (B) When scytalone was added to the culture medium, cells of the albino transformant eventually became black like the wild type (indicated by arrow).

The derived amino acid sequence of ElPks1p suggested it had multiple domains, orthologous with other type I Pksp [14]. BLAST analysis with the predicted ElPks1p amino acid sequence showed that it contained the expected domains for a β-ketoacyl synthase, an acyl transferase, two acyl carrier proteins and a thioesterase (Fig. 3). Disruption of *ElPKS1* with pCQx4 (Fig. 4A), which was constructed by ligation of a 1.5-kb fragment from the thioesterase domain with the pCB1004-derived plasmid, instead of with pCQ700, produced a second type of mutant, which was brown instead of albino (Fig. 5A). DNA sequence analysis of the two mutant types showed that when the integrations with pCQ700 were in the coding region for the acyl transferase domain, melanin synthesis ability was lost and the transformants were albino (e.g., *elpks1*Δ-*A1*). However, when the integrations with pCQx4 were in the coding sequence of the thioesterase domain, then transformants were brown (e.g., *elpks1*Δ-*B1*), and not black like the wild type, suggesting that their ability to synthesize melanin was only partially affected (Fig. 5A). Scytalone additions to a brown strain (*elpks1*Δ-*B1*) also turned it black like the wild type (Fig. 5B).

3.3. Cloning and characterization of ElPKS2

To isolate *ElPKS2*, the 500-bp PCR product amplified from this gene was used as a probe to screen a *Sac*I- and *Sac*II-digested partial genomic DNA library by colony

Fig. 3. Alignments of the active sites of the Type 1 multiple domains of ElPks1p, ElPks2p, and WdPks1p by using CLUSTAL-W analysis. The conserved active site residues important for enzyme function are indicated by arrows and the domains identified are for the β-ketoacyl synthases, acyl transferases, acyl carrier proteins, and thioesterases. Consensus symbols in the consensus line: "*" indicates identical residues in all sequences in the alignment; and ":" indicates conserved substitutions.

hybridization. Nine putative clones were identified, among which two (pCQpk5 and pCQpk2) were mapped, subcloned, and sequenced. The smaller clone (pCQpk5) contained only a portion of *ElPKS2*, whereas the larger clone (pCQpk2), contained the whole *ElPKS2* gene and considerable flanking sequences with additional genes (Fig. 6A). The *ElPKS2* sequencing involved 7672 bp, which included the UTS, an ORF coding for 2155 amino acids, four introns, which were all confirmed by RT-PCR and by RT-PCR product sequencing (Table 1), and a termination sequence. BLAST comparisons of the derived amino acid sequence showed that ElPks2p also represented a type I polyketide synthase and contained the four signature motifs (Fig. 3). However, disruptions with vector pCQ61 (Fig. 6B), which was constructed by ligation of a 0.9-kb fragment of *ElPKS2* with pCB1004 and linearized by *Nar*I before conducting electroporation, and Southern analysis (Fig. 6C) provided strong evidence that this Pksp was not involved in melanin biosynthesis. Most notably, the *elpks2*Δ mutants all were black like their wild-type parent (data not shown), instead of being albino or brown like the *elpks1*Δ mutants.

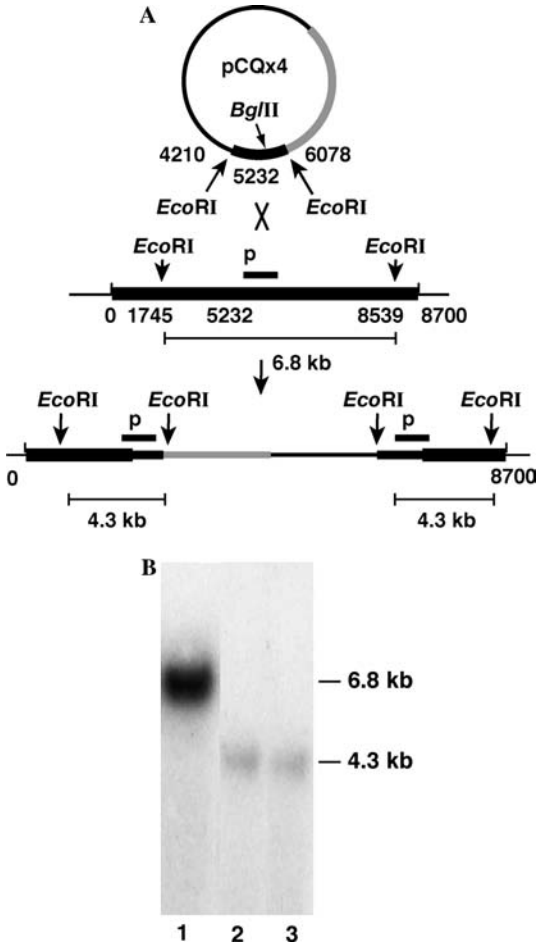

Fig. 4. Strategy of specific insertion of pCQx4 into the *ElPKS1* thioesterase locus and Southern analysis confirmation of the disruption. (A) Disruption plasmid pCQx4 was constructed by ligation of a 1.5-kb fragment from *ElPKS1* thioesterase-encoding domain into a pCB1004-derived plasmid, which also contained a bacterial catechol 2,3-dioxygenase gene expression cassette. The vector was linearized with *Bgl*II before the transformation of *E. lecanii-corni*. (B) Southern hybridization analysis of DNA of the wild type (lane 1), and two brown *elpks1*Δ-*B* strains (*B1* and *B2*, lanes 2 and 3, respectively) digested with *Eco*RI and probed (p) with a 1.5-kb *Sal*I fragment of *ElPKS2*.

BLAST analysis with the deduced amino acid sequences of either *ELPKS1* or *ELPKS2* showed that they both shared a high degree of sequence similarity with 13 other fungal polyketide synthases in the GenBank database. CLUSTAL-W analysis of those derived proteins showed (Fig. 7) that a minimum of four groups with defined functions were represented. Those involved in DHN-melanin biosynthesis ([15,22–24], AF395534 direct GenBank submission) comprised the largest group with seven members and were associated with *ElPK*sp. The second group with two members was comprised of those involved in green pigment (naphthopyrone)

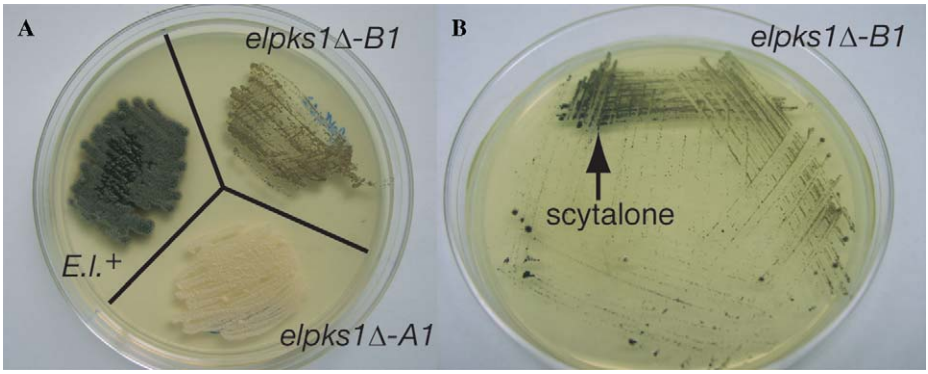

Fig. 5. Phenotype of *elpks1*Δ-*B* mutants. Comparisons of the *E. lecanii-corni* (*E.l.*$^{+}$) wild type (black), *elpks1*Δ-*A1* (white), and *elpks1*Δ-*B1* (brown) strains grown on YPD agar medium without (A) scytalone and with (B) scytalone added to the medium. Note that the *elpks1*Δ-*B1* brown strain in (B) became black like the wild type.

production [25–27]. The third group, consisting of three members, is responsible for aflatoxin and sterigmatocystin biosynthesis ([21,28], AB076803 direct GenBank submission). The fourth group consisted of a single member, which is involved in bikaverin biosynthesis (AJ278141 direct GenBank submission).

ElPks2p, which had less than 40% amino acid identity with the other fungal Pksps, appeared not to belong to any of these groups, as was the case for one other pksp with an unknown function (AB072444 direct GenBank submission). Taken together these results strongly suggested that the product of *ElPKS2* is involved in a polyketide biosynthesis pathway, but not one for melanin biosynthesis or any other known biosynthetic pathway. Support for this hypothesis was provided by preliminary TLC results that indicated that ElPks2p might be responsible for the synthesis of a non-polar compound, which had an R_f of about 0.78, and was present in extracts of the wild type, but was lost from those of the *elpks2*Δ mutant (data not shown). Efforts are continuing to identify the compound, whose biosynthesis is affected by the disruption of *ElPKS2*. The genes flanking *ElPKS2* (Fig. 6A) were putatively identified as ones that encode a transposase, a laccase, and a salicylate monooxygenase. The latter two enzymes may be involved in tailoring the PKS-derived product, but their identities did not shed further light on the nature of this product.

3.4. Comparisons of the growth characteristics of the wild type and the elpksΔ strains

Because *E. lecanii-corni* can grow on very simple synthetic media composed of only a few mineral salts and a carbon source [5], we chose to compare the growth rates of our two types of *elpks*Δ mutants with those of the wild-type parent in the nutritionally rich medium YPD and a simple defined medium (BM) with glucose or 4-hydroxybenzoic acid as the sole source of carbon. The purpose of these particular studies was to confirm that the *ElPKS* disruptions did not significantly affect the yeast morphology (data not shown) or the growth kinetics of the fungus, which in

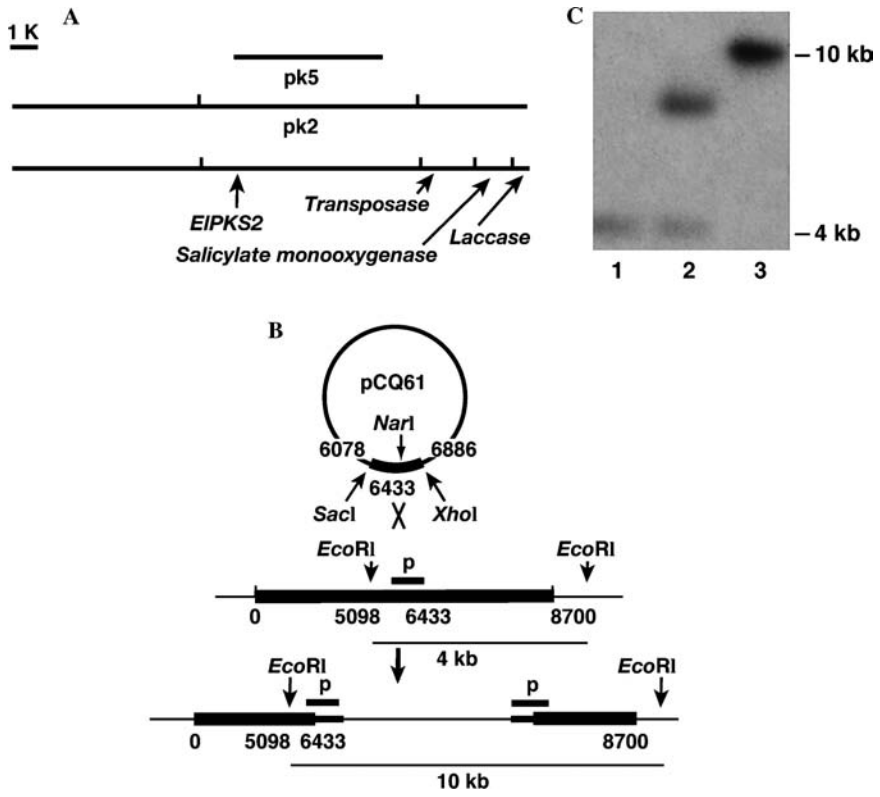

Fig. 6. Identification of *ElPKS2* in DNA fragments, the *ElPKS2* disruption strategy, and the Southern analysis of an *elpks2*Δ mutant. (A) Localization of the *ElPKS2* gene in pCQpk5 and the genes for *ElPKS2*, a glutamate permease, transposase-like structure, a salicylate monooxygenase and a laccase in the 20-kb fragment from pCQpk2. (B) Strategy for the disruption of *ElPKS2* with pCQ61. C. Southern blots of the wild type (lane 1), a transformant with an ectopic integration (lane 2) and an *elpks2*Δ mutant (lane 3).

turn might adversely affect the usefulness of such mutants in bioremediation applications. The results showed that in YPD and BM with glucose, the two mutants grew at essentially the same rates as their wild-type parent (Table 2). However, the generation times of the two mutants were somewhat longer than that of the wild type in BM supplemented with 4-hydroxybenzoate.

4. Discussion

Melanins represent a diverse group of pigments, which play an assortment of roles in bacteria, fungi, plants, and animals. Because of their numerous biological roles and their involvement in disease states, melanins have become the focus of many recent investigations. Melanins produced by pathogenic as well as non-pathogenic fungi are of interest for a variety of reasons. While not essential for fungal growth,

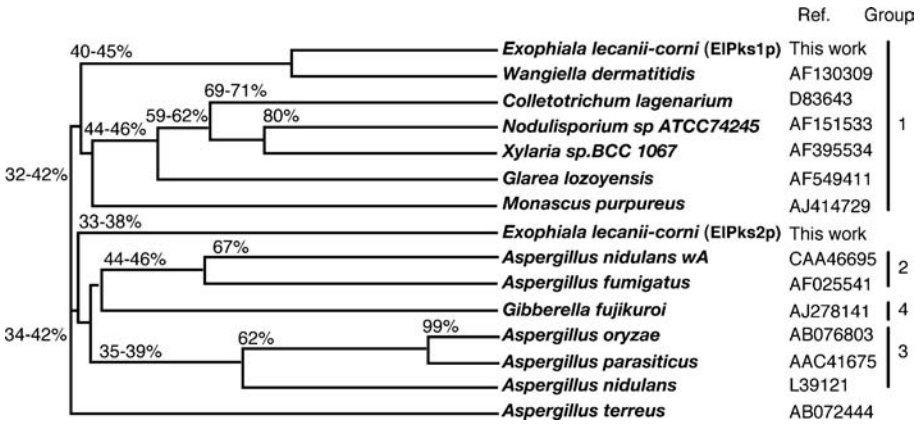

Fig. 7. CLUSTAL-W dendogram showing the general relationships of the deduced amino acid sequences of ElPks1p and ElPks2p with those of 13 other fungal, type I Pksps. The numbers at the nodes represent the maximum percent identities exhibited by each paired individual or group. The branch lengths do not necessarily indicate rigorous calculations of evolutionary distances or phylogenetic relationships. However, phylogenetic relationships are generally reflected by the closer associations among the Pksp involved in the same functions among species known or suspected to be more closely related.

Table 2
Comparison of the generation time (G.T.)[a] of the wild type and *elpks* mutants in different media

Strains	Media		
	YPD	BM + G	BM + HBA
	G.T. (h) ± SD[b]	G.T. (h) ± SD	G.T. (h) ± SD
Elwt	7.50 ± 0.174	7.89 ± 1.183	7.38 ± 0.280
*elpks1*Δ	7.38 ± 0.100	7.50 ± 0.396	9.14 ± 0.614
*elpks2*Δ	7.41 ± 0.223	7.48 ± 0.240	8.36 ± 0.231

[a] Generation time: represents growth status of log-phase cells during culture from 24 to 72 h.

melanin is often associated with the virulence of pathogenic fungi and contributes to the survival of both pathogenic and non-pathogenic fungi in stressful environments [9,11,29]. One of the major biosynthetic routes for the production of fungal melanins involves a polyketide synthase, which generates the THN building block. Subsequent enzymatic tailoring of the THN molecule and oxidation of the resulting 1,8-DHN affords the melanin.

The results of the present work are entirely consistent with these general observations and confirm that melanin synthesis in *E. lecanii-corni* occurs by the 1,8-DHN route. We have shown that *ElPKS1* is responsible for the generation of the 1,3,6,8-THN precursor because disruption of the gene blocks melanin production (as evidenced by the albino colonies) while the addition of scytalone enables the mutant to make melanin, as indicated by the black colonies. We have also shown that the disruption of *ElPKS1* results in no apparent changes in the growth characteristics of *E. lecanii-corni*.

These observations indicate that the *ElPKS1* genomic locus will be an appropriate site for the insertion of native and foreign genes for improving the usefulness of *E. lecanii-corni* as an agent for VOC remediation of waste gas streams. Moreover, the bio-engineered colonies are readily screened by their albino color. To this end, the bacterial catechol 2,3-dioxygenase gene from *Pseudomonas putida* mt-2 [30] under the regulation of the *glaA* promoter from *Aspergillus awamori* (kindly provided by Dr. M. Ward, Genencor, Palo Alto, CA) has been ligated into pCB1004 with a fragment of the *ElPKS1* gene to target the thioesterase-encoding region and used in *E. lecanii-corni* transformations as an initial test of the system. Three transformants with brown color have been identified and confirmed by RT-PCR to have been expressed successfully in *E. lecanii-corni* (Q. Cheng, S.C. Wang, C.K. Gunsch, K.A. Kinney, C.P. Whitman and P.J. Szaniszlo. Abstr. 103rd Gen. Meet. Am. Soc. Microbiol. 2003, abstr. Q297, p.568, 2003). Additional tests to confirm the production of a functional protein from this gene are underway.

Multiple *PKS* genes in the same fungus have been reported for *Glarea lozoyensis* [24] as well as *Aspergillus nidulans* [25,28]. In *Glarea lozoyensis*, three genes have been reported although only one, designated *pks1*, has been characterized and shown to be involved in the production of THN [24]. Characterization of the remaining two genes may assist in the identification of the gene product for *ElPKS2*, but such a characterization has not yet been reported. In *Aspergillus nidulans*, the *wA* gene, which encodes a Pksp required for the synthesis of a green pigment present in the walls of its conidia, has been extensively described [25,31,32], as has another *PKS* gene involved in the biosynthesis of the toxin sterigmatocystin [28,33]. Based on these observations, we carried out a TLC experiment aimed at showing that *ElPKS2* might be involved in the production of a similar compound. However, our TLC results produced no evidence that *E. lecanii-corni* had the ability to produce the most common aflatoxins or sterigmatocystin (data not shown), although it did produce a non-polar compound that dissolved in chloroform and was not produced by the *elpks2*Δ mutants. Nonetheless, our finding of genes for a monooxygenase and a laccase near *ElPKS2* suggests that this gene resides in a chromosomal region that encodes proteins possibly involved in a number of similarly complicated biosynthetic or degradative processes. However, the situation is not straightforward, because of our finding of a transposase-like-encoding gene between *ElPKS1* and the flanking monooxygenase gene. To our knowledge, no such gene has been identified near any *PKS*-related gene cluster [14,26,33,34].

Our sequence comparisons of the 15 fungal type-I *PKS* genes suggested that their derived proteins sort into as many as six groups. Of these, four have defined function for the biosynthesis of melanin, naphthopyrone, toxins or bikaverin. The largest of these groups included ElPks1p and consisted of six additional polyketide synthases, five of which had previously been shown to be required for DHN-melanin biosynthesis in other fungi. Among these ElPks1p was no less than 40% identical at the amino acid level with all the others, and was most identical (82%) with WdPks1p, the type 1 Pksp for melanin biosynthesis in *W. dermatitidis* (Fig. 7), a result that would not be unexpected because of the close relationship of the two species involved. These data

further support our conclusion that ElPks1p is required for melanin biosynthesis in *E. lecanii-corni*.

In contrast, ElPks2p does not appear to be very closely related to any of the remaining sequences. This observation suggests that ElPks2p is involved in a new biosynthetic pathway. Experiments are underway to elucidate the nature of this pathway. Because ElPks2p does not seem to be essential to the growth of *E. lecanii-corni*, its gene locus, like that of ElPks1p, may provide another site for the insertion of native and foreign genes. Experiments to test this possibility are also being designed.

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