Lovastatin biosynthesis in *Aspergillus terreus*: characterization of blocked mutants, enzyme activities and a multifunctional polyketide synthase gene

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Background: Lovastatin, an HMG-CoA reductase inhibitor produced by the fungus *Aspergillus terreus*, is composed of two polyketide chains. One is a nonaketide that undergoes cyclization to a hexahydronaphthalene ring system and the other is a simple diketide, 2-methylbutyrate. Fungal polyketide synthase (PKS) systems are of great interest and their genetic manipulation should lead to novel compounds.

Results: An *A. terreus* mutant (BX102) was isolated that could not synthesize the nonaketide portion of lovastatin and was missing a ~250 kDa polypeptide normally present under conditions of lovastatin production. Other mutants produced lovastatin intermediates without the methylbutyryl sidechain and were missing a polypeptide of ~220 kDa. The PKS inhibitor cerulenin reacted covalently with both polypeptides. Antiserum raised against the ~250 kDa polypeptide was used to isolate the corresponding gene, which complemented the BX102 mutation. The gene encodes a polypeptide of 269 kDa containing catalytic domains typical of vertebrate fatty acid and fungal PKSs, plus two additional domains not previously seen in PKSs: a centrally located methyltransferase domain and a peptide synthetase elongation domain at the carboxyl terminus.

Conclusions: The results show that the nonaketide and diketide portions of lovastatin are synthesized by separate large multifunctional PKSs. Elucidation of the primary structure of the PKS that forms the lovastatin nonaketide, as well as characterization of blocked mutants, provides new details of lovastatin biosynthesis.

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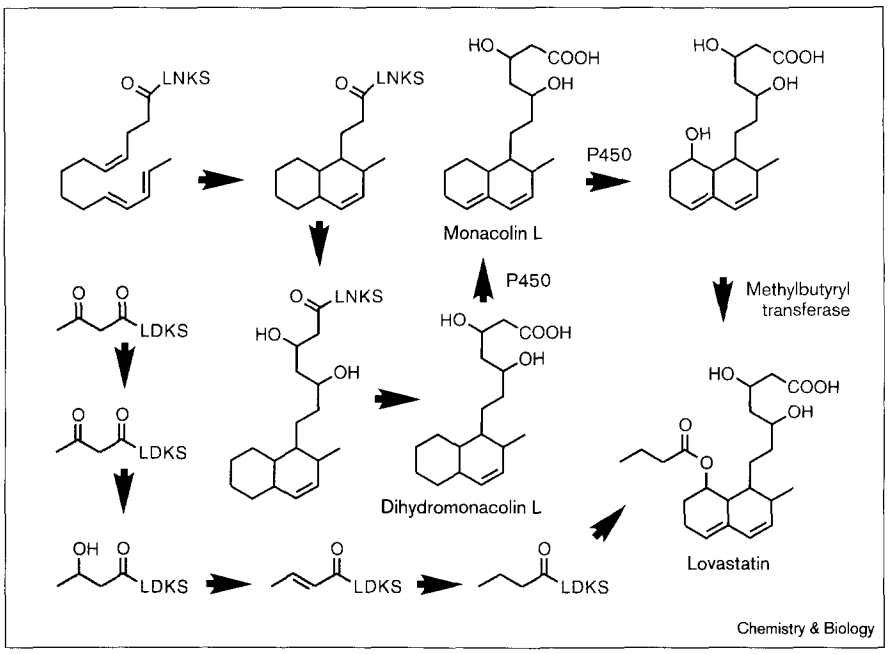
Introduction

Lovastatin (also known as mevinolin, Mevacor and monacolin K), an inhibitor of HMG-CoA reductase produced by *Aspergillus terreus* and other fungi, is used to reduce serum cholesterol levels in humans [1]. It is biosynthetically derived from two polyketide chains joined through an ester linkage [2–5]. One chain is the diketide 2-methylbutyrate and the other is a nonaketide that includes a distinctive conjugated hexahydronaphthalene ring system.

Over the past several years it has become clear that polyketides are assembled in a variety of mechanistically complex ways [6]. Polyketide synthases (PKSs) are structurally and functionally related to the fatty acid synthases (FASs), both catalyzing sequential decarboxylative condensations between ACP-linked acyl-thioesters. Unlike most FASs, however, PKSs can omit some or all of the reduction reactions (β-keto reduction, dehydration and enoyl reduction) that take place after each condensation, thereby yielding products with ketone, alcohol or alkene instead of methylene functional groups at specific positions along the chain. There are at least three types of PKS systems [6]: the iterative type II systems of actinomycetes, which generally produce aromatic structures such as actinorhodin or tetracenomycin; the modular type I systems of actinomycetes, which produce macrolides such as erythromycin; and the iterative type I systems typical in fungi, which produce precursors to widely different structures such as patulin, sterigmatocystin or lovastatin.

Figure 1 shows an overall scheme for lovastatin biosynthesis, incorporating data from isotope labeling studies [2–5], biotransformation of intermediates [7–9] and this work. Important details of this pathway, such as the C-methylation reactions, the transition from reduced to more oxidized carbons in the nonaketide and cyclization to the unusual hexahydronaphthalene ring system, proposed to involve a Diels–Alder condensation [2–5], have remained poorly understood. Studies of lovastatin biosynthesis should provide new insights into the complex structure–function relationships of iterative multifunctional PKS systems. As a first step towards elucidating the enzymology

Figure 1



Hypothetical pathway for lovastatin biosynthesis. Independent pathways form the diketide (blue) and nonaketide (green) portions of lovastatin, which we propose are catalyzed by lovastatin diketide synthase (LDKS) and lovastatin nonaketide synthase (LNKS), respectively. Both enzymes build their polyketide product from an acetyl starter unit and malonyl extender units, and both add a methyl group from S-adenosyl methionine (shown in yellow). The intermediates shown bound to the PKSs are hypothetical, but consistent with results described here. Cyclization via a Diels–Alder condensation is not proven, but is the current hypothesis. The oxygen in red is derived from a P450 oxidation. Details shown in this figure are discussed in the text.

of lovastatin biosynthesis, we isolated mutants of *A. terreus* that no longer produced lovastatin. Using these mutants, the gene for the PKS involved in synthesis of the nonaketide portion of lovastatin was cloned and characterized, suggesting new details of lovastatin biosynthesis.

Results

Isolation of mutants blocked in lovastatin biosynthesis

In a mutagenesis and screening effort aimed at increasing lovastatin titer, *A. terreus* mutants were found that did not produce lovastatin. In this effort lovastatin and its biosynthetic intermediates were resolved using high-performance liquid chromatography (HPLC) and quantitated by absorbance at 239 nm. The mutants were derived from a lovastatin-producing strain designated CW. Five mutants produced monacolin J at about 60% the level of lovastatin-produced by strain CW, and low levels of monacolin L. These mutants appeared at a higher than expected frequency, suggesting a mutational hot spot. This phenotype indicates a mutation in a gene encoding either synthesis of the methylbutyryl group or its transfer to monacolin J to give lovastatin. One mutant was isolated that produced monacolin X (lovastatin with a methyl-acetoacetyl sidechain instead of a methylbutyryl sidechain, as first observed in cultures of wild-type *Monascus ruber* [10]), plus monacolin J, the titers being about 40% and 10%, respectively, of the lovastatin titer produced by strain CW. This phenotype most probably results from a defect in the ketoreductase domain of the lovastatin PKS.

Another mutant was identified that produced no detectable metabolite with the characteristic UV absorbance spectrum of lovastatin intermediates. This mutant (BX102) produced other metabolites derived from the polyketide emodin, as reported for lovastatin production strains [11], indicating that they had not suffered a general loss of secondary metabolite production. Moreover, if cultures of BX102 were fed monacolin J, they efficiently converted it to lovastatin, indicating that the genes for synthesis and attachment of the methylbutyryl group were functionally expressed. Strain BX102 was therefore defective in a gene encoding an enzyme catalyzing the synthesis of the nonaketide portion of lovastatin. Because mutants were defective in synthesis of either the diketide or the nonaketide without affecting the other pathway, the biosynthetic pathways for these two portions of lovastatin are independent and utilize separate enzymes. We call the corresponding PKSs lovastatin nonaketide synthase (LNKS) and lovastatin diketide synthase (LDKS).

Identification of lovastatin biosynthetic activities in cell-free extracts

To assay lovastatin PKSs, cell-free extracts were incubated in a reaction mixture that included [^{14}C]malonyl-CoA, acetyl-CoA, S-adenosyl methionine, NADPH and ATP. The labeled products were extracted with ethyl acetate, resolved using HPLC and counted for ^{14}C incorporation. Extracts of strain CW incorporated radioactivity into components eluting with the retention times of lovastatin carboxylate, lovastatin lactone and palmitic acid, whereas extracts of strain BX102 grown under the same conditions incorporated label into the peak co-eluting with palmitic acid, but not into lovastatin (Figure 2). The labeled products co-eluting with lovastatin carboxylate

and lovastatin lactone were isolated and shown to be converted into the other form by incubation at pH extremes. We therefore showed that *in vitro* synthesis of lovastatin was occurring in cell-free extracts. Incorporation of labeled malonyl-CoA into lovastatin did not occur if any of the cofactors (including ATP) were omitted. Cerulenin (at 1 mM), a specific inhibitor of polyketide and fatty acid synthases [12], completely inhibited incorporation of [^{14}C]malonyl-CoA into both lovastatin and palmitic acid.

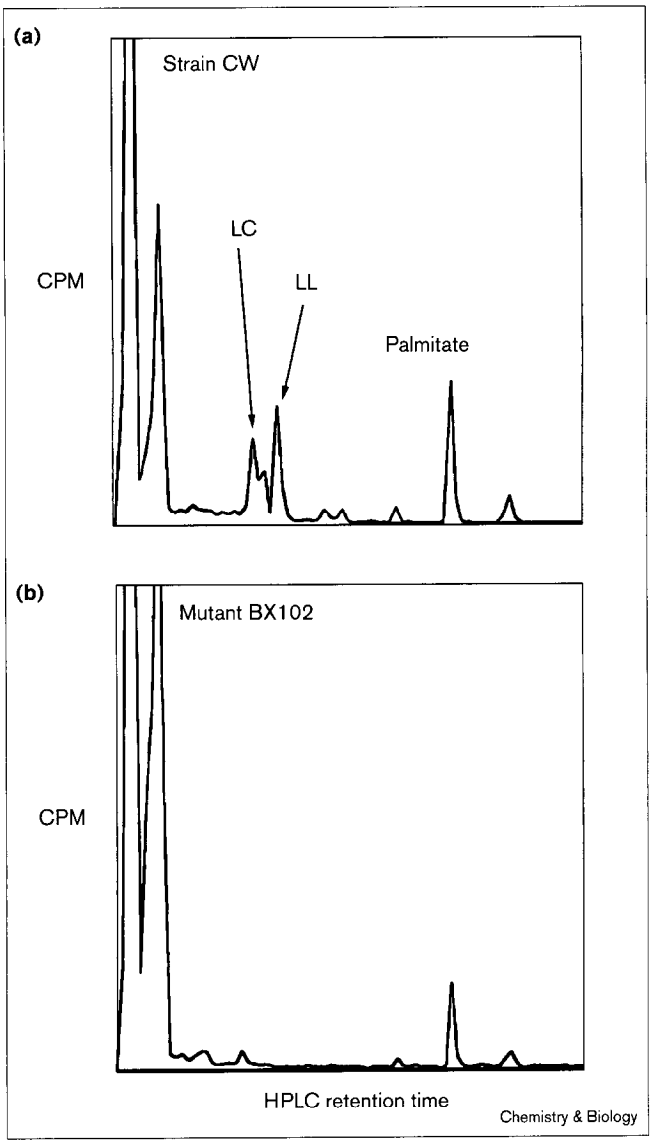
In attempts to purify the predicted lovastatin PKSs, crude extracts were subjected to several types of chromatography and fractions were assayed for incorporation of [^{14}C]malonyl-CoA into ethyl acetate extractable products, as detected by autoradiography of thin-layer chromatograms. Although particular column fractions did show NADPH-dependent incorporation of malonyl-CoA into specific ethyl acetate extractable products, indicative of possible lovastatin PKS activities, we were unable to identify the structure of those products. Moreover, the partially purified activities were not stable and were difficult to further purify. This result also has been encountered with other polyketide systems and researchers have relied on molecular analyses of mutants blocked in the pathway [6].

Identification of polypeptides missing in the mutants

Crude extracts prepared from strains CW and BX102 grown under production conditions gave nearly identical profiles of polypeptides using Coomassie-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), with the exception of an ~250 kDa polypeptide that was absent in strain BX102. The ~250 kDa polypeptide was also present in a monacolin-J-producing mutant (PS10) grown under production conditions, but was absent from cultures in the preproduction phase of growth. To visualize better these large polypeptides, organomercurial agarose chromatography was used to partially purify them. Protein eluted from such resin with 100 mM dithiothreitol was resolved using preparative SDS–PAGE. The ~250 kDa polypeptide was extracted from the gel and used to raise rabbit polyclonal antiserum. By comparing different strains using this enrichment procedure and analyzing polypeptides using both stained SDS–PAGE and western blots, we were able to show that the ~250 kDa polypeptide was completely absent from the BX102 mutant and that a different polypeptide of about 220 kDa was absent from the monacolin-J-producing mutants (Figure 3). Rabbit polyclonal antiserum was also raised against this ~220 kDa polypeptide and used to verify that it was absent in the monacolin-J-producing mutants (data not shown).

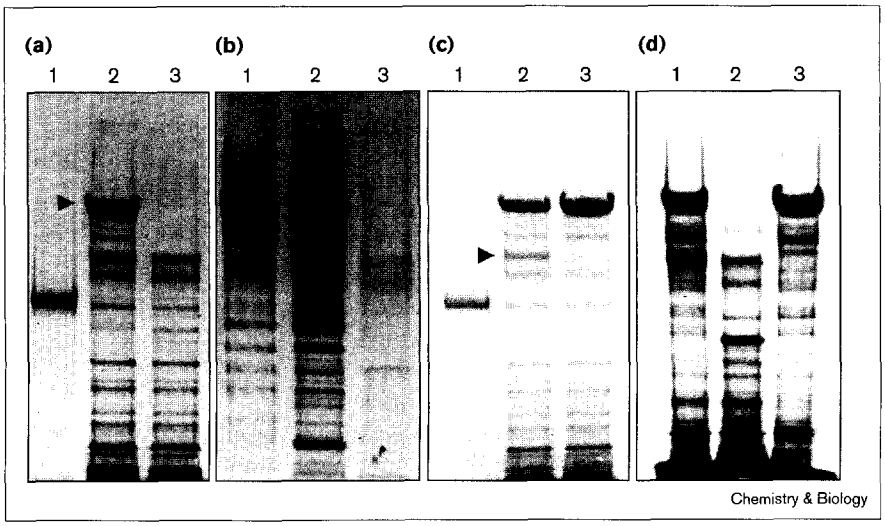
Cerulenin inactivates polyketide and fatty acid synthases by forming an irreversible covalent bond with the active-site cysteine in the ketoacyl synthase domain [12,13]. As mentioned above, cerulenin inhibits lovastatin synthesis *in vitro*. When fractions enriched for PKSs using organomercurial agarose chromatography were incubated with [^{3}H]cerulenin and resolved using SDS–PAGE, and the labeled proteins subsequently detected by autofluorography of the gels, several polypeptides, including those of ~220 and ~250 kDa, became labeled (Figure 3). In agreement with results from the stained gels and western blots, both the ~220 and ~250 kDa polypeptides were present in strain CW, whereas strain PS10 was specifically missing the ~220 kDa polypeptide and strain

Figure 2



Cell-free extracts from the lovastatin-production strain, but not the nonproducing mutant, can synthesize lovastatin *in vitro*. Cell-free extracts from **(a)** strain CW and **(b)** mutant BX102 were incubated with [^{14}C]malonylCoA and other reaction components as described in the Materials and methods section. The reaction products were extracted with ethyl acetate and resolved using HPLC. Fractions were collected and the radioactivity was determined using liquid scintillation counting. LC, lovastatin carboxylate; LL, lovastatin lactone.

Figure 3



Mutants blocked in the lovastatin pathway are missing specific high molecular weight polypeptides that covalently bind cerulenin. **(a)** A Coomassie-dye-stained gel of extracts from production strain CW (lane 2) and mutant BX102 (lane 3) after enrichment by thiol agarose chromatography and ultracentrifugation (see the Materials and methods section). The ~250 kDa polypeptide missing in mutant BX102 is indicated with an arrowhead. Lane 1 is a 200 kDa protein standard (rabbit skeletal muscle myosin). **(b)** A western blot in which the gel was blotted to nitrocellulose, reacted with anti-250 kDa polypeptide antiserum and developed using alkaline phosphatase conjugated secondary antibody. Lane 1 is the gel-purified ~250 kDa polypeptide, lane 2 is the extract from strain CW and lane 3 is the extract from mutant BX102. The lower molecular weight bands in lanes 1 and 2 that cross react with the antiserum are proteolytic fragments of the ~250 kDa polypeptide, because they are also seen in the purified protein preparation but do not correspond to any of the prominently staining bands present in the extract. **(c)** A Coomassie-dye-stained gel of organomercurial resin-enriched fractions from strain CW (lane 2) and a triol-producing mutant (PS10) (lane 3). Lane 1 is the 200 kDa protein standard. The ~220 kDa polypeptide in lane 2 missing in mutant PS10 is indicated by the arrowhead. **(d)** An autofluorograph of a gel in which organomercurial resin-enriched fractions from strains CW (lane 1), BX102 (lane 2) and PS10 (lane 3) were incubated with [^{3}H]cerulenin for 60 min and the labeled polypeptides resolved using SDS–PAGE. In all these experiments the gels were precast 4% acrylamide gels from Novex run under identical conditions. The position of a given polypeptide band in the four gel images is therefore similar.

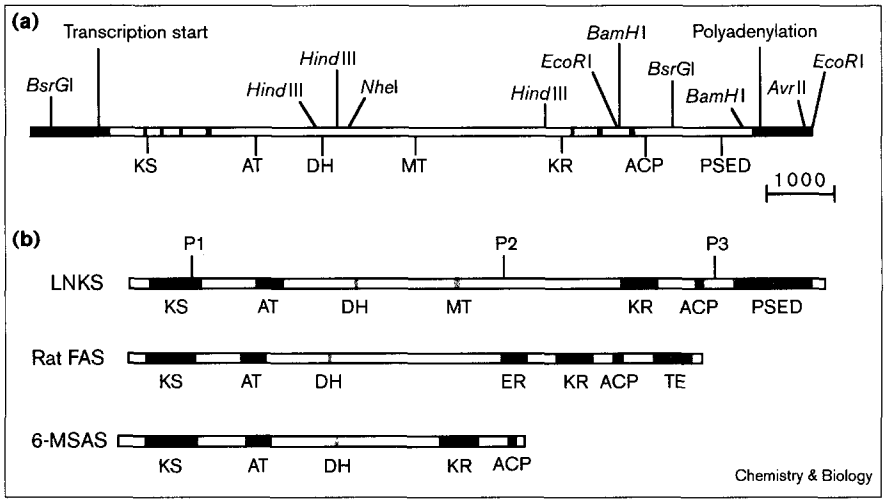
BX102 was missing the ~250 kDa polypeptide, as well as another polypeptide of ~230 kDa. Mutant BX102 also contained a strongly labeled polypeptide of ~140 kDa not observed in strains CW or PS10. It is unlikely that this ~140 kDa cerulenin-labeled polypeptide is a fragment of either the ~220 or ~250 kDa polypeptides, as it was not observed on a western blot. It may represent another PKS (see below), or a proteolytic fragment thereof, that can only interact with cerulenin in the absence of the PKS represented by the ~250 kDa polypeptide. These data suggest that the ~250 and ~220 kDa polypeptides represent PKSs for synthesis of the nonaketide and diketide portions of lovastatin, respectively. The additional polypeptides that interact with cerulenin probably represent β-ketoacyl synthases, or enzymes catalyzing similar reactions, other than those required for lovastatin biosynthesis. A 6-methylsalicylic acid synthase (6-MSAS) gene has been reported in *A. terreus* [14] and there should be a PKS for the synthesis of emodin (the precursor to sulochrin produced by *A. terreus* [11]), plus possibly a PKS for synthesis of spore pigment, as seen in *A. nidulans* [15].

Cloning and sequencing the gene for the ~250 kDa polypeptide

Initial attempts to clone lovastatin PKS genes by hybridization to the ketoacyl synthase domain of the *Penicillium patulum* 6-MSAS gene [16] resulted only in cloning an *A. terreus* 6-MSAS homolog. Genomic DNA from strain CW was therefore used to prepare a cosmid library in an *Aspergillus/Escherichia coli* shuttle cosmid (pLO9), and the library was used to complement the BX102 mutation. Out of ~6000 transformants of mutant BX102, one lovastatin producer was identified, from which the cosmid was recovered by *in vitro* packaging of its genomic DNA [17]. Four of 20 isolates transformed with this recovered cosmid were restored for production of lovastatin and the ~250 kDa polypeptide, as analyzed using SDS–PAGE. Some transformants may not express the large PKS gene either because it is disrupted during the integration event [18] or because integration occurs at a silent chromosomal locus.

In another approach, cDNA libraries prepared in the *E. coli* vector λgt11 using mRNA from a lovastatin-producing culture were screened with the antiserum raised against the ~250 kDa polypeptide. Using this approach, 18 clones were isolated, several of which encoded one of two different ubiquitin homologs, possibly because the ~250 kDa polypeptide used to prepare the antiserum had been ubiquitinated. Two cDNA clones were found that hybridized to the BX102-complementing cosmid clone described above, however. The longer one (cDNA2-9 that has a 2 kilobase (kb) insert) was found to contain a polyA tail, a 3′-untranslated region, and an open reading frame encoding the carboxyl terminus of a protein with an acyl carrier protein motif similar to those of *P. patulum* 6-methylsalicylic acid synthase [16] and rat fatty acid synthase [19]. The cDNA sequence also encoded the first of three amino acid sequences previously obtained from

Figure 4



Structure of the LNKS gene and protein. **(a)** A map of the LNKS gene region with 5′ and 3′ noncoding regions shown in black and introns shown in red. Also shown are the locations of transcription start, polyadenylation and the catalytic sites (see below). **(b)** The conserved sequence motifs in LNKS compared with those of rat fatty acid synthase and *P. patulum* 6-methylsalicylic acid synthase. Color-coded regions represent catalytic domains for β-ketoacyl synthase (KS; 42% identity with rat FAS over 231 residues), acetyl/malonyl transferase (AT; 30% identity with rat FAS over 126 residues), dehydratase (DH; motif HALQGQTVFPAAG), methyl transferase (MT; see Figure 6), enoyl reductase (ER; not found in LNKS), β-ketoreductase (KR; 33% identity with rat FAS over 171 residues), acyl carrier protein (ACP; 31% identity with rat FAS over 45 residues) and the peptide synthetase elongation domain (PSED; see Figure 6). The positions of three peptide sequences (see text) obtained from the purified ~250 kDa polypeptide are indicated above the LNKS sequence (P1, P2 and P3).

prominent lysC protease fragments of the ~250 kDa polypeptide (VLGGASITDLANEAA, TDRDANLFPT and TGITMPN; using single-letter amino acid code).

The cDNA2-9 clone was used to isolate a set of overlapping λEMBL3 clones from an *A. terreus* genomic library, and the sequence of an 11.6 kb region was obtained. In addition, cDNA sequence was obtained from fragments amplified by the polymerase chain reaction (PCR) from a total cDNA pool using primers designed from the genomic sequence. Figure 4 shows the structure of the isolated gene, which we name lovastatin nonaketide synthase (LNKS). The gene contains seven short (53–81 base pairs; bp) introns having splice junction motifs typical of other fungal introns. Primer extension analysis revealed a predominant transcription initiation point, and a few minor ones nearby. Upstream of these were sequence motifs typical of eukaryotic promoters: the sequence TTAATA at –30 bp (relative to the transcription start) and the sequence CAAT at –80 bp. The predicted mRNA transcript is ~10 kb in length and encodes a 3038 amino acid protein with a predicted molecular weight of 269 kDa. This size agreed well with the estimated apparent size from SDS–PAGE (~250 kDa). All three amino acid sequences obtained from proteolytic fragments of the ~250 kDa polypeptide were encoded in the gene.

Lovastatin titer is affected by the expression level of the LNKS gene

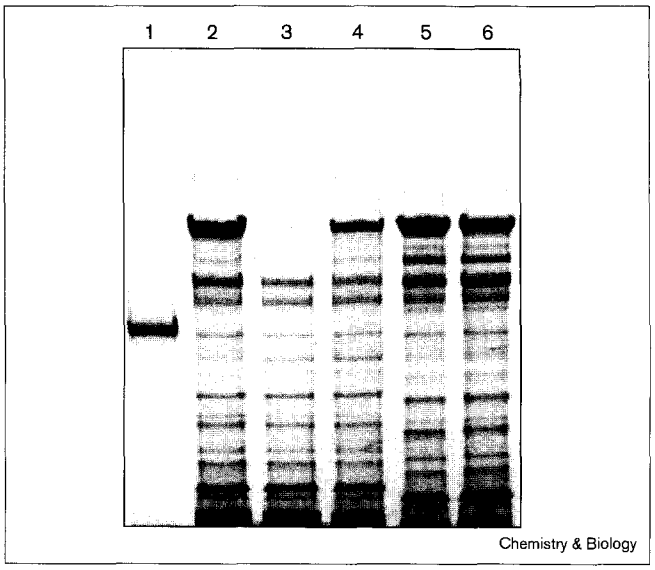
Transformation of mutant BX102 with pLO9 containing the cloned LNKS gene as an 11 kb fragment restored lovastatin production, proving the direct involvement of this PKS gene in lovastatin production. The level of lovastatin produced by different transformants correlated with the level of the ~250 kDa polypeptide accumulated in those strains (Figure 5), suggesting that the abundance of LNKS polypeptide in the production strains is important for the high-production phenotype.

Structural features of the lovastatin nonaketide PKS protein

Figure 4 shows the relative positions of functional domains identified in LNKS compared with rat fatty acid synthase [19,20] and *P. patulum* 6-methyl salicylic acid synthase [16]. The first third of LNKS contains conserved β-ketoacyl synthase, acyl transferase and dehydratase domains, spaced similarly between the three enzymes. The β-ketoacyl synthase and acetyl/malonyl transferase domains of LNKS are highly similar to their counterparts from other enzymes. The dehydratase domain is clearly identified in the nonaketide PKS by its characteristic motif [21,22]. The β-keto reductase and acyl carrier protein motifs of the nonaketide PKS are also well conserved and are spaced similarly as for other FASs and PKSs. As several carbons of dihydromonacolin L are fully reduced, a functional enoyl reductase (ER) domain was expected in LNKS. Although sufficient space was present in the LNKS sequence to accommodate an ER domain, no sequence motifs typical of such domains were identified.

There are two sequence motifs found in the nonaketide PKS that are particularly noteworthy (Figure 6). One is a putative S-adenosyl methionine binding motif found between the putative dehydratase and enoyl reductase domains. Because S-[^{13}C]methyl methionine labels the C6 methyl group of lovastatin [2–4], an adenosyl-methionine-dependent methyl-transferase activity must be involved. Although methyl-transferase domains have

Figure 5



LNKS gene expression in transformants of mutant BX102 correlates with lovastatin production. Shown is a Coomassie-stained SDS gel of protein extracts enriched by organomercurial resin chromatography. Lane 1 is a 200 kDa protein standard. Samples were prepared from strain CW (lane 2), mutant BX102 (lane 3), and three transformants of BX102 in which lovastatin production was restored (lanes 4–6). Relative to CW the level of lovastatin produced by these strains was 0%, 33%, 73% and 54%, respectively.

been seen in multifunctional peptide synthetases [23,24], we have not seen a report that identifies such a motif in a multifunctional PKS. The other motif of interest is at the carboxy-terminal end of the nonaketide PKS where, instead of a thioesterase domain, there is a domain with strong homology to the elongation domains of peptide synthetases, which catalyze peptide-bond formation. As there is no known role for amino acids, or other nitrogen-containing compounds, in lovastatin biosynthesis, the role of this carboxy-terminal domain of LNKS remains to be determined.

Homologs of lovastatin nonaketide PKS in other fungi

Several other fungi produce lovastatin-related structures, including *M. ruber*, which produces lovastatin [25], and *Penicillium citrinum* and *P. brevicompactum*, which produce compactin [26,27]. Compactin is identical to lovastatin except that it is missing the methionine-derived methyl group on the nonaketide. Genomic DNA from these fungal species was analyzed using Southern blot hybridization to the ketoacyl synthase region of LNKS gene (Figure 7). Hybridization was to a single genomic region in *A. terreus*, *M. ruber* and *P. citrinum*, indicating that in each of these strains there is only one gene closely related to LNKS. Blots containing DNA from *P. brevicompactum* gave a complex pattern of hybridization that we could not interpret. A probe from *A. terreus* cDNA clone 2–9 consisting of the acyl carrier protein and peptide synthetase domains of LNKS gave similar results. In contrast, a probe consisting of the putative methyl-transferase domain hybridized only to DNA from *A. terreus* and *M. ruber*, but not to DNA from *P. citrinum* or *P. brevicompactum*. This is consistent with the fact that these latter two species do not carry out the methylation reaction.

Discussion

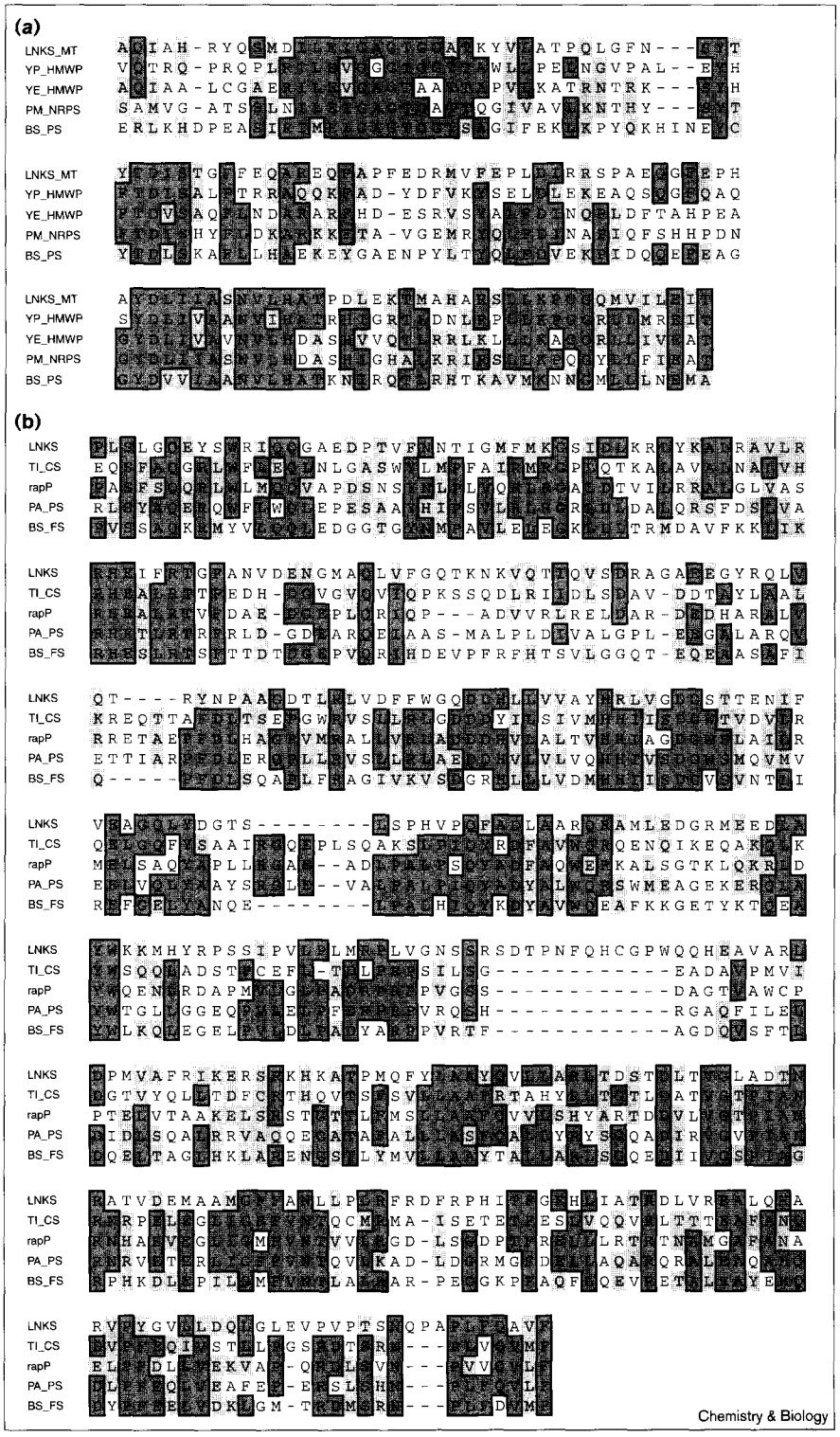
Isotopic labeling studies [2–4] and *in vivo* biotransformation of putative pathway intermediates [7–9] led to a hypothetical scheme for lovastatin biosynthesis. The results reported here are consistent with this scheme, and have suggested new enzymological details, as shown in Figure 1. The gene for a large (~250 kDa) polypeptide was cloned and found to encode a type I PKS that can complement mutant BX102, indicating that it was the proposed LNKS gene. A polypeptide of ~220 kDa, which reacts covalently with cerulenin and is missing in mutants unable to synthesize the methylbutyryl group, probably represents LDKS, but definitive proof of this will require isolation of this gene. LNKS contains sequence motifs for catalytic functions expected for the synthesis of the nonaketide chain of lovastatin, and these are arranged in the same order as seen in other multifunctional polyketide and fatty acid synthases. Although there is sufficient space for an enoyl reductase domain at the amino-terminal end of the KR domain, a sequence signature motif representative of an ER was not seen. If LNKS does not encode an ER activity, this has important implications for the mechanism of synthesis of dihydromonacolin L. LNKS also has motifs that represent homologs of methyl transferases and the elongation domain of peptide synthetases, as discussed below.

Biosynthesis of each polyketide chain of lovastatin includes one C-methylation reaction [2–4]. Because we found a mutant that accumulates monacolin X, C-methylation of the diketide presumably occurs prior to keto reduction, when the flanking carbonyls should facilitate carbanion formation. The existence of this mutant suggests that the activity normally responsible for the transfer of the methylbutyryl group to monacolin J also is capable of transferring the methylacetoacetyl group. The significant amount of monacolin J produced by this mutant suggests, however, that transfer of a methylacetoacetyl group from LDKS is less efficient than transfer of a methylbutyryl group, consistent with the substrate specificity seen for the putative methylbutyryl transferase *in vitro*.

The C-methylation required for the biosynthesis of monacolin J [2–4] may be an integral function of LNKS on the basis of the presence of a methyl-transferase signature sequence in LNKS. Although such a domain has not been reported in other PKSs, they are present and functional in several nonribosomal peptide synthetases

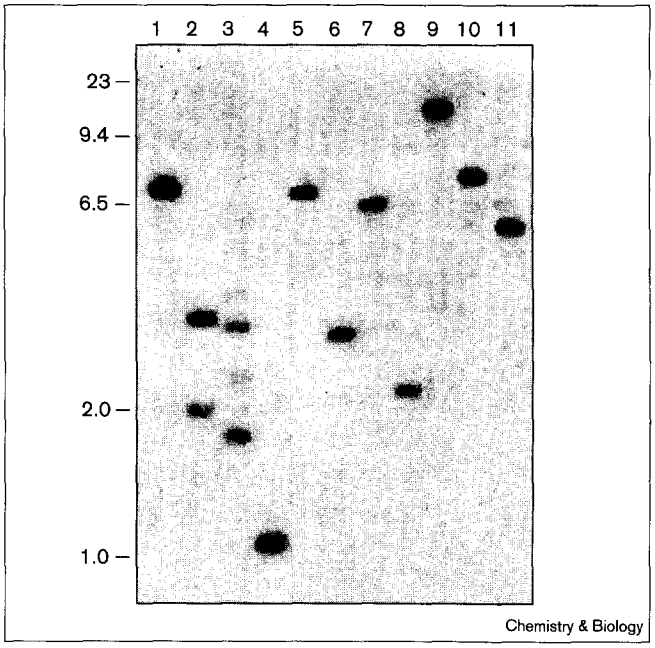
Figure 6

Domains in LNKS homologous to methyltransferase and peptide synthetase elongation domains. Sequences were aligned using default settings of the CLUSTAL algorithm in MacVector (Oxford Molecular). **(a)** Methyltransferase sequences, with GenBank accession numbers, are: YP_HMWP, *Yersinia pestis* high molecular weight protein (AF091251); YE_HMWP, *Yersinia enterolyticus* high molecular weight protein (Y12527); PM_NRPS, *Proteus mirabilis* nonribosomal peptide synthetase (U46488); BS_PS, *Bacillus subtilis* polyketide synthase (Z99113). **(b)** Peptide synthetase elongation domain sequences are: TI_CS, *Tolypocladium inflatum* cyclosporin synthetase (Z32674); rapP, rapamycin pipecolate incorporating enzyme from *Streptomyces hygroscopicus* (X86780); PA_PS, *Pseudomonas aeruginosa* pyoverdine synthetase (AF002222); BS_PS, *Bacillus subtilis* fengycin synthetase (AF023465).



[23,24]. In addition, BLAST searches with this sequence found a hypothetical polyketide synthase in the *Bacillus subtilis* genome (see Figure 6), which also has a methyltransferase signature sequence. It is reasonable to propose that this domain catalyzes methylation of the lovastatin nonaketide chain during its assembly. Functional dissection of this domain might be achieved by expressing chimeras of the *A. terreus* LNKS gene and the compactin

Figure 7



Potential homologs of the LNKS gene in other fungi. Shown is an autoradiogram of a genomic Southern blot using a probe consisting of a gel-isolated 1 kb *Pst*I fragment from the LNKS gene labeled by random oligonucleotide priming. The position of DNA size markers on the gel is indicated on the left. Lanes 1–4, *A. terreus* DNA cut with *Sal*I, *Sac*I, *Pvu*II and *Pst*I, respectively. Lanes 5–8, *M. ruber* DNA cut with *Sal*I, *Sac*I, *Pvu*II and *Pst*I, respectively. Lanes 9–11, *P. citrinum* DNA cut with *Sal*I, *Sac*I and *Pvu*II, respectively.

nonaketide synthase gene of *P. citrinum*, which should not have a functional methylation domain.

An interesting feature of the nonaketide portion of lovastatin is the relatively reduced state of the first six acetate units in the chain, and the relatively unreduced state of the last three. Formation of the hexahydronaphthalene ring system has been postulated to involve an intramolecular Diels–Alder condensation [2–5]. If the nonaketide PKS is solely responsible for synthesis of dihydromonacolin L, this enzyme represents one of the most sophisticated iterative PKSs known. Aside from the additional domains to catalyze the C-methylation and Diels–Alder condensation, the enzyme apparently uses its dehydratase domain selectively. Does this depend on chain length, or perhaps the Diels–Alder condensation? Yoshizawa *et al.* [3] have proposed a possible reaction sequence for dihydromonacolin L in which the chain is partially synthesized, the Diels–Alder condensation occurs and finally the chain is completed. Although we were unable to identify any of the several products produced by the partially purified PKS fractions, none comigrated with dihydromonacolin L, the putative first intermediate in the nonaketide pathway [7–9]. Recently, Hutchinson, Vederas and their colleagues [28] have determined the structure of molecules produced by *A. nidulans* heterologously expressing the *A. terreus* LNKS gene. Their work suggests that LNKS might produce a precursor of dihydromonacolin L that requires at least one other enzyme to complete the synthesis.

Following the acyl carrier domain, the nonaketide PKS contains an unusually long carboxy-terminal region that shares significant homology to elongation domains of peptide synthetases. These domains presumably catalyze attack of the acyl thioester on one phosphopantetheine by the amino group of an amino acid on an adjacent phosphopantetheine [23,24]. Such a domain also might catalyze condensation or transfer through a nucleophile other than an amino group, however. There is considerable parallelism between polyketide and nonribosomal peptide synthesis [29] and there are examples of molecules, such as rapamycin and bleomycin, that are constructed of both amino acid and acyl extender units.

Hutchinson and colleagues [28] have recently sequenced the lovastatin biosynthetic gene cluster of *A. terreus* and have identified several genes surrounding the LNKS gene with roles in lovastatin biosynthesis. We have previously identified an enzyme that may catalyze transfer of the methylbutyryl group from LDKS to monacolin J and a candidate gene for this enzyme was found in the cluster. In sterigmatocystin biosynthesis transfer of a hexanoyl chain from a specialized fatty acid to a PKS has been proposed [20]. The PKS completes the chain, skipping all reductions to give a putative polyketide intermediate that is fully reduced at one end and fully unreduced at the other [30,31]. The N-acetyl cysteamine derivative of hexanoic acid is efficiently incorporated into the sterigmatocystin precursors, but not if one of the genes for the specialized FAS is disrupted [30]. This implies interaction between the FAS and the PKS for labeling to occur and also suggests transfer of the hexanoyl group from the FAS to the PKS. Among the lovastatin biosynthetic genes found by Hutchinson and coworkers, none is a candidate for a third PKS that might 'finish' the monacolin L chain by a mechanism similar to that just described. Instead, LNKS may undergo a conformational (or covalent) change prior to the last two acyl condensations, in order to ensure that only keto reduction occurs after those final condensations.

Significance

The manipulation of polyketide biosynthetic genes has provided novel structures, many of potential pharmaceutical value, that would be difficult and expensive to obtain by synthetic chemistry. Fungal polyketide synthases (PKSs) have been less well studied than the PKS systems of actinomycetes despite the fact that they have unique features and produce unusual polyketides. The mechanistic enzymology of fungal PKSs is interesting, particularly how such enzymes use their catalytic sites

selectively depending on the structure of the product during its iterative assembly. This work paves the way for detailed studies leading to an understanding of the biosynthesis of an economically important natural product, lovastatin. We have provided evidence that the lovastatin nonaketide synthase contains catalytic domains not previously seen in PKSs: one for C-methylation and one for an activity related to nonribosomal peptide synthesis.

Materials and methods

Strains and culture conditions

The *A. terreus* strains used in this work were derived from an industrial strain development program described previously [11,32]. Strains were maintained on YME media (0.4% yeast extract, 0.1% malt extract, 0.4% glucose, 5 ppm $FeSO_4 \cdot 7H_2O$, 2% agar, pH 7.2). A two-stage shake flask procedure for lovastatin production was used, as described previously [11,32]. Lovastatin was extracted from cultures with two volumes of ethanol followed by dilution with methanol and filtering prior to analysis. Radiolabeled lovastatin and lovastatin precursors were prepared by adding [^{14}C]glucose to resting cultures of selected strains and isolating, using semipreparative HPLC, the desired compounds. Resting cultures were prepared by washing and resuspending mycelia from a production culture in 50 mM Tris, pH 7.5 to 3× the original density.

Analytical methods

HPLC analysis of metabolites from *A. terreus* used a Waters system with a C18 Novapak radial compression cartridge and photodiode array detector. Mobile phases (at 1.5 ml/min) were A: acetonitrile with 0.02% trifluoroacetic acid and B: distilled water with 0.02% trifluoroacetic acid. Initial conditions were 35% A, 65% B. After sample injection, there was a 3 min linear gradient from initial conditions to 65% A, 35% B followed by 4 min at those conditions. Lovastatin and related metabolites were detected by their absorbance maximum at 238 nm. A reference standard of lovastatin was provided by Merck. Monacolin J was prepared from lovastatin by hydrolysis in 0.1 M LiOH at 95°C. The identity of monacolin X and monacolin L was verified using ^{13}C and proton NMR spectrometry.

Preparation of cell-free extracts and enzyme assays

Mycelia from production cultures were harvested on Miracloth, washed with cold water, lyophilized and pulverized in extraction buffer (20 mM Tris pH 8.0, 10% glycerol, 1 M NaCl, 5 mM ascorbate, 1 mM EDTA, 1 mM DTT, 3.8 μg/ml leupeptin, 17.7 μg/ml chymostatin, 2.0 μg/ml pepstatin, 0.2 mM PMSF, 2.2% polyvinyl polypyrrolidone) by grinding with glass in a mortar and pestle. The homogenate was centrifuged at 10,000 g for 10 min to give a crude cell-free extract. PKS and FAS activities were assayed in crude or partially purified protein fractions by incubating with a labeled substrate and other required factors, extracting with ethyl acetate, and analyzing the labeled products using TLC or HPLC. The standard reaction mixture was 50 μl containing 50 mM Tris, pH 7.5, 0.1 μM [^{14}C]malonyl-CoA ($\sim 4 \times 10^5$ cpm), 300 μM acetyl-CoA, 1 mM S-adenosyl methionine, 0.1 mM NADPH, and 0.1 mM ATP. After incubation at 28°C for 1 h, the resulting products were extracted twice with ethyl acetate, samples were dried, taken up in methanol, and products resolved using HPLC (see above) or using chromatography on thin layer silica plates developed with a solvent of 90% CH_2Cl_2 and 10% methanol containing 1 M acetic acid and 1 M ammonium acetate. Radioactive products on TLC plates were visualized by autoradiography, whereas radioactivity in HPLC fractions was determined by scintillation counting.

Isolation of high molecular weight polypeptides

Crude cell-free extract was prepared as described earlier except the lyophilized mycelia was first washed with acetone. For analytical experiments crude extract was applied directly to an organomercurial agarose (Bio-Rad Affi-gel 501) column equilibrated in Buffer A (20 mM Tris, pH 8; 50 mM NaCl; 5 mM EDTA; 5 mM ascorbic acid). The column was washed with 5 vol buffer A, 10 vol buffer A containing 0.5 M NaCl, and 5 vol buffer A. Bound proteins were eluted with 40 ml buffer A containing 100 mM DTT. Samples were concentrated and dialyzed for SDS–PAGE analysis. For preparation of polypeptide antigen, proteins were precipitated from the crude extract with 16% PEG 3,000 followed by centrifugation at 30,000 g for 30 min and applied to an organomercurial agarose (Bio-Rad Affi-Gel 501) column. Protein eluted from the column was subjected to ultracentrifugation at 180,000 g for 16 h. The supernatant was discarded and the protein pellet dissolved in gel loading buffer consisting of 125 mM Tris, pH 6.8; 10% glycerol; 0.005% bromphenol blue; 2% SDS; 0.6 M 2-mercaptoethanol and heated to 95°C for 10 min. The sample was electrophoresed on a preparative 1.5 mm, 4% acrylamide SDS gel (Novex) at 140V for 2 h using electrode buffer consisting of 25 mM Tris; 192 mM glycine; 0.1% SDS. Electrophoresis was stopped when the prestained 200 kDa reference standard was 1.4 cm from the bottom of the gel. To visualize proteins the gel was rinsed briefly with water, soaked in 0.2 M imidazole for 10 min, transferred to 0.3 M zinc acetate for 5 min and finally rinsed with water. Stained bands of the desired size were excised from the gel and destained in 0.25 M Tris, 0.25 M EDTA, pH 9.5 for 5 min until the slice became transparent.

To generate antisera destained gel slices were crushed between glass plates and forced sequentially through 18 and 25 gauge needles. A 0.5 ml aliquot of this material was mixed with an equal volume of Freund's complete adjuvant and injected into rabbits. Boosts at 21 and 42 days used material prepared as described, but with 0.5 ml Freund's incomplete adjuvant. Antibody was affinity purified by immobilizing the antigen on PVDF membrane by electro-transfer from a preparative SDS polyacrylamide gel. The area of the membrane containing the polypeptide of interest was cut out, incubated in TTBS (50 mM Tris, pH 7.5; 0.5 M NaCl, 0.05% Tween 20) plus 5% non-fat dry milk for 1 h, and washed 3× for 5 min in TTBS. The immobilized antigen was incubated for 5 h with 2 ml of antisera diluted 1:1 with TTBS plus 1% non-fat dry milk. The membrane was washed 4× 10 min with TTBS, and the bound antibody was eluted with 2 ml of 0.1 M glycine, pH 2.8. The eluted antibody was neutralized with 50 μl of 1.0 M Tris, pH 9.5.

High molecular weight polypeptides were resolved using SDS–PAGE (Novex 1 mm 4% acrylamide gels). For western blots, proteins were electrophoretically transferred to nitrocellulose in 25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol, 0.05% SDS at 240 mA for 2 h. Blots were rinsed for 1 min in TTBS then blocked for 2 h in TTBS with 5% non-fat dry milk. Incubation with primary antibody was for 16 h at a 1:1000 dilution of antisera in TTBS containing 1% nonfat dry milk. The blot was washed in TTBS 3× for 5 min each and incubated with goat antirabbit alkaline phosphatase conjugate diluted 1:1000 for 2 h in TTBS containing 1% nonfat dry milk. After washing 4× 10 min in TTBS, color development was achieved with 5-bromo-4-chloro-3 indolyl phosphate (115 μg/ml) and nitroblue tetrazolium (330 μg/ml) in 66 mM Tris, pH 9.5; 0.1 M NaCl; 5 mM $MgCl_2$.

Isolation of nucleic acids

To isolate genomic DNA, lyophilized mycelia were ground dry with sand in a mortar and pestle, mixed with lysis buffer (100 mM NaCl; 50 mM EDTA; 10 mM Tris, pH 8.0; 1% SDS; 50 μg/ml pancreatic RNase; 50 μg/ml Proteinase K) and extracted with an equal volume of Tris-saturated phenol-chloroform by gently shaking for 1 h at 37°C. Upon centrifugation at 8,000 g for 10 min, the extraction was repeated with phenol–chloroform, followed by chloroform. DNA was precipitated with ethanol and resuspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

To isolate RNA, production culture mycelia was collected on Miracloth, washed with distilled water and quickly frozen in liquid nitrogen. Frozen mycelia was ground to a fine powder with glass in a mortar and pestle under liquid nitrogen. The frozen powder was added to 50 mM Tris

pH 7.4, 150 mM NaCl, 5 mM EDTA, 5% SDS and the mixture extracted twice with phenol/chloroform. The final aqueous layer was brought to 3 M LiCl, kept at −20°C for 4 h, centrifuged at 12,000 xg for 20 min and the pellet resuspended in RNase-free water. Polyadenylated RNA was isolated by chromatography on oligo-d(T) cellulose according to the manufacturer's instructions (Poly(A)Quik™ mRNA purification kit from Stratagene).

Library construction

The cosmid library was prepared in pLO9 (see below) by ligation to separate left and right arms [33], packaged using the GigaPak Gold kit (Stratagene) and transfected into *E. coli* DH5α. pLO9 was constructed from pUC18, a modified *A. niger* β-tubulin promoter fragment, the phleomycin resistance gene plus yeast CYC1 gene terminator cassette from pUT713 (Cayla), and a PstI fragment from pBTI-1 (Boehringer-Mannheim) containing the λcos site. To prepare cosmid DNA with full representation for transforming BX102, *E. coli* cells carrying the library were plated at confluence, cells were scraped from the plates in 10 ml of cold 50 mM Tris pH 8.0, 10% sucrose, and DNA was isolated by gentle SDS lysis and purified by CsCl density gradient centrifugation [33]. Vector DNA with insert was isolated from vector DNA without insert by resolution on 0.5% agarose gels. The phage genomic library was prepared using predigested EMBL3 arms (Promega), packaged with GigaPak Gold (Stratagene), and transfected into *E. coli* LE392.

Polyadenylated RNA was used to construct two cDNA libraries in λgt11 using the Superscript Choice System (BRL) according to the manufacturer's instructions. First strand synthesis was primed using either 0.05 μg random hexamers plus 0.5 μg oligo(dT)12–18 or 1 μg oligo(dT)12–18 alone. *E. coli* strain Y1090 was used as the host. To decrease cross reaction to *E. coli* proteins the antisera was pre-reacted with a lysate of Y1090 cells and this antiserum was used to screen the λgt11 library [33].

Sequencing

Raw sequence data was generated using an Applied Biosystems 373A automated DNA sequencer according manufacturer's protocols. Genomic clones were sequenced from randomly selected clones of 1–2 kb fragments and by oligonucleotide primer walking. cDNA was sequenced from PCR products generated using primers designed from the genomic sequence. The sequence was assembled using the SeqMan program (Lasergene package from DNA Star) on a Macintosh computer.

Transformation of A. terreus

To prepare spheroplasts of *A. terreus*, 10^8 spores were inoculated into 50 ml CM media in a 250 ml flask and cultures were grown for 30 h at 200 rpm and 28°C. Mycelia was collected on Miracloth and 4 g was suspended in 100 ml KMP (700 mM KCl, 800 mM mannitol, and 20 mM potassium phosphate, pH 6.3). *Trichoderma harzianum* lysing enzymes (100 mg; Sigma) were added and the flask was shaken at 100 rpm for 18 h at 28°C. Spheroplasts were filtered through Miracloth into 50 ml conical centrifuge tubes, concentrated by centrifugation at 100 g, and washed twice by suspending in 15 ml of KCM (700 mM KCl; 10 mM MOPS adjusted to pH 5.8) and centrifuging. Washed spheroplasts were suspended at 5×10^7/ml in KCMC (KCM brought to 50 mM $CaCl_2$ just prior to use). For transformation, 6.5 μl of KCMC containing 5 units of heparin was added to a tube containing 5 μg of vector DNA in 20 μl TE. The spheroplast suspension (200 μl) was added, followed by 50 μl of PCM (40% PEG-8000, 10 mM MOPS, pH 5.8, 50 mM $CaCl_2$) and the suspension incubated on ice for 30 min. The spheroplast suspension was diluted with 0.6 ml PCM, transferred to 50 ml of melted MA (5% Clutterbuck's salts, 0.5% tryptone, 0.5% yeast extract, 1.0% glucose, 23% mannitol, 2% agar) maintained at 45°C, and immediately distributed into five tared petri dishes. After incubation at 28°C for 4 h, each plate was overlayed with an equal volume of overlay agar (1% peptone, 1% agar, 100 μg/ml phleomycin) and incubated at 28°C until sporulating colonies could be picked (7–10 days).

Accession numbers

The accession number of the LNKS sequence is Genbank AF151722.

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