University of Wollongong

Research Online

Faculty of Health and Behavioural Sciences -
Papers (Archive)

Faculty of Science, Medicine and Health

2010

Molecular cloning and characterization of the global regulator LaeA in *Penicillium citrinum*

Wei Xing
Southwest University

Chao Deng
University of Wollongong, chao@uow.edu.au

Chang-Hua Hu
Southwest University

Follow this and additional works at: <https://ro.uow.edu.au/hbspapers>

Part of the [Arts and Humanities Commons](#), [Life Sciences Commons](#), [Medicine and Health Sciences Commons](#), and the [Social and Behavioral Sciences Commons](#)

Recommended Citation

Xing, Wei; Deng, Chao; and Hu, Chang-Hua: Molecular cloning and characterization of the global regulator LaeA in *Penicillium citrinum* 2010.
<https://ro.uow.edu.au/hbspapers/3460>

Research Online is the open access institutional repository for the University of Wollongong. For further information contact the UOW Library: research-pubs@uow.edu.au

Molecular cloning and characterization of the global regulator LaeA in *Penicillium citrinum*

Abstract

We have cloned and analysed a *laeA* gene (*Pci-laeA*) that may control mevastatin biosynthesis in *Penicillium citrinum*. The full-length *Pci-laeA* sequence is 1,340 bp with an ORF of 1,284 bp encoding 427 amino acids. It shows 95% identity with *LaeA* from *P. chrysogenum*. The predicted molecular mass of *Pci-LaeA* is 48.72 kDa with an estimated theoretical isoelectric point of 6.96. *Pci-LaeA* has a conserved S-adenosylmethionine binding site and a potential MlcR (a pathway specific regulator in mevastatin biosynthesis) binding site.

Disciplines

Arts and Humanities | Life Sciences | Medicine and Health Sciences | Social and Behavioral Sciences

Publication Details

Xing, W., Deng, C. & Hu, C. (2010). Molecular cloning and characterization of the global regulator *LaeA* in *Penicillium citrinum*. *Biotechnology Letters*, 32 (11), 1733-1737.

Molecular cloning and characterization of the global regulator LaeA in *Penicillium citrinum*

Wei Xing¹, Chao Deng², Chang-Hua Hu^{1,2,*}

1. Institute of Modern Biopharmaceuticals, School of Pharmaceutical Sciences, Southwest University, Beibei, Chongqing 400716, People Republic of China
2. School of Health Sciences, University of Wollongong, Wollongong, 2522, NSW, Australia

*Corresponding author.

Prof Chang-Hua Hu, Institute of Modern Biopharmaceuticals, School of Pharmaceutical Sciences, Southwest University, Beibei, Chongqing 400716, People Republic of China

Fax: +86 23 68251225

E-mail address: chhu@swu.edu.cn (C-H. Hu)

The section to be selected: Microbial and enzyme biotechnology

Abstract

LaeA, a novel global regulator of secondary metabolism, has been discovered in filamentous ascomycetes. In this study, we have cloned and analysed a *laeA* gene (*Pci-laeA*) which may control mevastatin biosynthesis in *Penicillium citrinum*. The full-length *Pci-laeA* sequence is 1340 bp in size, and contains an open reading frame of 1284 bp encoding 427 amino acid, which shows 95% identity with that of *P. chrysogenum* LaeA found in 2008. The predicted molecular mass of Pci-LaeA is 48.72 kDa with an estimated theoretical isoelectric point of 6.96. Pci-LaeA shows a conserved S-adenosylmethionine binding site and a potential MlcR (a pathway specific regulator in mevastatin biosynthesis) binding site.

Keywords: Cloning; Global regulator LaeA; Mevastatin; *Penicillium citrinum*

Introduction

Mevastatin (compactin), the first potent and specific competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, is a polyketide compound produced by *Penicillium citrinum*, and has been used as a substrate for microbial conversion to pravastatin, a widely used pharmaceutical product for the therapy of hypercholesterolemia (Endo *et al.* 1976, Park *et al.* 2003). The gene cluster responsible for compactin biosynthesis in *P. citrinum* has been identified (Abe *et al.* 2002). Among them, *mlcR* is a pathway-specific zinc cluster transcriptional activator of the compactin biosynthetic genes (Baba *et al.* 2009).

In addition to pathway-specific regulators, fungal secondary metabolite biosynthesis is also regulated by global regulators (Keller *et al.* 2005). LaeA, a global regulator for morphological development and secondary metabolism in filamentous fungi has been identified (Bok and Keller 2004). In *Aspergillus nidulans*, where LaeA was first discovered, deletion of *laeA* resulted in a loss of production of numerous secondary metabolites, such as sterigmatocystin and penicillin (Bok and Keller 2004). Moreover, the production of several toxins was regulated by LaeA in *Aspergillus fumigatus* (Bok *et al.* 2005). In *Penicillium chrysogenum*, LaeA positively regulated penicillin biosynthesis, the formation of pigmentation and sporulation (Kosalkova *et al.* 2008). Therefore, it is important to determine the role of *laeA* in the secondary metabolism of the mevastatin producer *P. citrinum*. In this work, we identified and characterized a *P. citrinum laeA* (*Pci-laeA*) gene encoding a protein very similar to that of *Penicillium chrysogenum*, *A. fumigatus* and *A. nidulans*. The discovery of *Pci-laeA* will facilitate elucidation of the regulatory mechanisms in mevastatin biosynthesis and contribute to strain improvement and the discovery of novel natural products.

Materials and Methods

Strains, media and culture conditions

Penicillium citrinum IMB002, a high-level mevastatin producer, was collected in our laboratory.

P. citrinum liquid cultures were initiated by inoculating fresh spores inoculated into complex medium (10 g tryptone/l, 50 g glucose/l, 30 g soybean powder/l, 10 g glycerol/l, 5 g NaNO₃/l, 1 g MgSO₄/l, pH 6.5). After incubation at 25 °C for 72h in an orbital shaker (210 rpm), aliquots were inoculated in complex mevastatin production medium CMPM (10 g tryptone/l, 50 g glucose/l, 35 g soybean powder/l, 50 g glycerol/l, 5 g NaNO₃/l, 1 g MgSO₄/l, 2.6 g bean oil/l, pH 6.5) at 25 °C.

Extract of genomic DNA and isolation of total RNA

Genomic DNA from *Penicillium citrinum* was isolated from mycelium grown for 96h in CMPM medium using the CTAB method. Total RNA was also isolated from mycelium grown for 96h in CMPM medium using the 'RNA-prep Pure Plant Kit' (Tiangen, China), following the manufacturer's instructions.

RT-PCR and cloning of the internal fragment of *P. citrinum laeA* cDNA

Based on the known amino acid sequences of several LaeA proteins deposited in the NCBI database, the conserved region of the *laeA* gene was identified by MegAlign program of DNASTAR software. Two degenerate primers D1: 5'-GARCARGAYCGYCTNGAYWT-3' and D2: 5'-GCYTGR-TAKATRTGMAG-3' were synthesized to amplify the internal fragment of *P. citrinum laeA* cDNA by RT-PCR using the 'Takara RNA PCR Kit' (Takara, Japan), following the manufacturer's instruction.

Cloning of 3' end sequences of the *laeA* using RACE technique

To obtain the 3'-end sequences of *laeA* cDNA, the rapid amplification of cDNA ends (RACE)

technique was used. The oligonucleotide d(T)18-adaptor reverse primer: 5'-TACCGTCGTTCCACTAGTGATTTCCTATAGGTTTTTTTTTTTTTTTTTTT-3' was used for the synthesis of the first-strand cDNA. The adaptor reverse primer: 5'-TACCGTCGTTCCACTAGTGATT-3' and the gene-specific primer F1: 5'-CCCGTGTTGAGGACAAGGATGGT-3' were used for amplification of cDNA. Subsequently, the PCR product was tested by two consecutive nested PCR reactions with the adaptor reverse primer and gene-specific primers F2: 5'-CTGATTCCCA CGAGCAGAAGGTT-3', and with the adaptor reverse primer and gene-specific primers F3: 5'-CAGTCGGGTTCTTTCTTGGTCC-3', respectively. .

Rapid isolation of 5'-flanking region of *laeA* by TAIL-PCR

Cloning of the 5' flanking region of *laeA* was carried out by TAIL-PCR (thermal asymmetric interlaced PCR) technique with some modifications (Liu and Whittier 1995): (1) 10 mers arbitrary primers AD1: 5'- GNANCANAGA-3', AD2: 5'- GNANCANAGG-3', AD3: 5'- CNGANANGAA-3' and AD4: 5'- GNACNTNGGA-3' were used; (2) 10 low-stringency cycles were deleted; and (3) the tertiary PCR procedure was the same as the secondary TAIL-cycling (15 super cycles) instead of normal cycles. Three gene-specific reverse primers R1: 5'-GTCAACACCGACCACGAAGGAAT-3', R2: 5'-GATGCTGAAAGACTCGTCGGTAG-3' and R3: 5'-CAAGAAAGAACCCGACTGAAAGG-3' were obtained against the cloned internal fragment of *laeA* cDNA.

Cloning of the whole *P. citrinum laeA* gene

Assembly of the internal and flanking fragments was carried out to form the full-length sequence of the *laeA* gene using the SeqMan program of DNASTAR software. The primers LAF1: 5'-AATATCAACGGATCCATGTCTTACAGAGAG-3' and LAR1: 5'-TCACCAGGG AGCTCTTATTCCTCGAGAGG-3' were used to amplify the coding region of *laeA* cDNA.

Bioinformatics analysis of the *P. citrinum* LaeA sequence

The molecular mass and pI (isoelectric point) of *P. citrinum* LaeA (Pci-LaeA) protein was determined using ProtParam tools (<http://www.expasy.org/tools/protparam.html>). The sequence homology was analyzed by BlastP of NCBI (<http://www.ncbi.nlm.nih.gov>). The alignment of amino acid sequences was carried out using the ClustalW2 (<http://www.ebi.ac.uk/clustalw/>). The prediction concerning subcellular localization of the protein was performed using WoLF PSORT software (<http://wolfpsort.org/>).

Accession number

The full length genomic DNA and cDNA sequences of the *Pci-laeA* gene were submitted to the GenBank database under the accession numbers GU647101 and GU906782 respectively.

Results and Discussion

Cloning and analysis of full length DNA and cDNA of *Pci-laeA*

Using degenerate primers D1 and D2, a 782-bp cDNA fragment was obtained from *P. citrinum* by RT-PCR (Fig. 1A). By 3'RACE and TAIL-PCR technique, we obtained the 3' end fragment (1136 bp) and a 5'-flanking fragment (981 bp) of *Pci-laeA* (Fig. 1B and Fig. 1C). The whole *laeA* gene (1340 bp) and the encoding gene *laeA* (1284 bp) gene were amplified from the genomic DNA and cDNA of *P. citrinum* by PCR using primers LAF1 and LAR1, respectively (Fig. 1D). A 56-bp intron spanning nucleotides 390–445 of the *Pci-laeA* gDNA were identified. Sequences of the 5' and 3' intron splice sites were 'GTATGT' and 'GAACAG' respectively. These concur with the consensus fungal intron splice sequences.

An examination of the *laeA* nucleotide sequence revealed one putative MlcR binding site

5'-ACGG-TTGTCGTGA-TCGG-3' that was very similar to the consensus sequence for the MlcR-binding sequence 5'-(A/T)CGG-NGTN3-6-TCGG-3' (Baba *et al.* 2009). It has been recognised that the mlcR binding sites motif is an asymmetric direct repeat that exists in the *mlcA-mlcC*, *mlcB-mlcD*, *mlcF-mlcG* and *mlcH-orf11* intergenic regions, and is essential for the transcriptional activation of *mlcA* and *mlcC* in *P. citrinum* (Baba *et al.* 2009). Other similar studies have revealed that another GAL-4 type pathway transcriptional activator AflR has binding sites in the sterigmatocystin (ST) cluster gene and *laeA* gene simultaneously in *A. nidulans* (Fernandes *et al.* 1998, Bok and Keller 2004). Overexpression of the *A. nidulans laeA* upregulated *afR* expression, while overexpression of *afR* downregulated *laeA* expression (Bok and Keller 2004). This implies that there may be a potential mlcR binding site in the *P. citrinum laeA* gene promoter and a unique interaction between *laeA* and mevastatin gene regulation.

Characterization of *P. citrinum* LaeA protein and homology analysis

The *Pci-laeA* encoded a protein of 427 amino acids with an estimated molecular mass of 48.72 kDa and a theoretical pI of 6.96. The results of sequence homology analysis indicated that Pci-LaeA showed 95%, 61% and 60% identity with LaeA protein of *P. chrysogenum*, *A. fumigatus* and *A. nidulans*, respectively. Alignment of the amino acid sequences of the Pci-LaeA protein revealed a conserved S-adenosylmethionine (SAM) binding motif (Fig. 2B), component of the 193rd to 200th amino acid residues (– LDLGCGTG –), the 219th and 239th amino acid residues of *P. citrinum* LaeA protein, normally found in most of the protein methyltransferase (Hamahata *et al.* 1996). Although Pci-LaeA contains the same SAM motif in histone methyltransferases and arginine methyltransferases as LaeA protein found in other species, it lacks two other conserved domains (SET domain and a double E loop). Therefore, Pci-LaeA is possibly a unique protein methyltransferase. This remains to be elucidated

through further study.

The Pci-LaeA protein was analyzed using WoLF PSORT software. The analysis showed that this protein was localized in the nucleus, however it did not contain obvious nuclear localization signal sequence. This phenomenon has also been observed in *A. nidulans* LaeA. In *A. nidulans*, the LaeA did not show the presence of a nuclear localization motif, however the existence of GFP tagging in either the 5' or 3' ends suggested that LaeA should be primarily localized in the nucleus (Bok and Keller 2004). Due to its nuclear localization and conserved methyltransferase SAM-binding motif, the Pci-LaeA protein should be correlated with a change in chromatin conformation (Bok and Keller 2004,).

The discovery of Pci-LaeA provides an important clue for understanding the secondary metabolism of mevastatin. As demonstrated by transcriptome microarray comparative analysis between wild-type, a LaeA deletion mutant and complemented control strains of *A. fumigatus*, LaeA modulates the expression of approximate 10% of the genes in *A. fumigatus*. Furthermore, 13 secondary metabolite clusters including 102 genes are under the control of LaeA (Perrin *et al.* 2007). Overexpression of *laeA* has been reported to enhance expression of some secondary metabolic gene clusters and consequently increased products formation. For example, there was 400% to 700% production increase in lovastatin when the *A. nidulans laeA* gene was heterogeneously expressed in the *A. terreus* (Bok and Keller 2004). In addition, over-expression of the *PclaeA* gene could lead to a 25% increase in penicillin production in *P. chrysogenum* (Kosalkova *et al.* 2008). It is even more exciting that modifications of LaeA have been used as a tool to trigger cryptic new natural products, such as terrequinone A (an anti-tumor agent that was not previously known to be produced by *A. nidulans*) (Bouhired *et al.* 2007). Although the function of LaeA has not been well understood, *laeA*-based genomic mining has already shown

strong potential as a strategy for activating silent cryptic biosynthetic gene clusters (Bok *et al.* 2006; Zerikly *et al.* 2009). The identification of *P. citrinum* LaeA will be useful for the improvement of industrial strain and the discovery of novel natural products.

Acknowledgments

This work was supported by the grants of Chongqing Key Program of Science and Technology Development (CSTC2009AB1029). We would like to thank Dr Mandy Reid and Dr Guojian Liao for critical reading of this manuscript.

References

- Abe Y, Suzuki T, Ono C, Iwamoto K, Hosobuchi M (2002) Molecular cloning and characterization of an ML-236B (compactin) biosynthetic gene cluster in *Penicillium citrinum*. *Mol Genet Genomics* **267**: 636–646
- Baba S, Kinoshita H, Hosobuchi M, Nihira T (2009) MlcR, a zinc cluster activator protein, is able to bind to a single (A/T)CGG site of cognate asymmetric motifs in the ML-236B (compactin) biosynthetic gene cluster. *Mol Gene. Genomics* **281**: 627–634
- Bok JW and Keller NP (2004) LaeA, a regulator of secondary metabolism in *Aspergillus* spp. *Eukaryot Cell* **3**: 527–535
- Bok JW, Balajee SA, Marr KA, Andes D, Nielsen KF, Frisvad JC, Keller NP (2005) LaeA, a regulator of morphogenetic fungal virulence factors. *Eukaryotic Cell* **4**: 1574–1582
- Bok JW, Hoffmeister D, Maggio-Hall LA, Murillo R, Glasner JD, Keller NP (2006) Genomic mining

- for *Aspergillus* natural products. Chem Biol **13**: 31–37
- Bouhired S, Weber M, Kempf-Sontag A, Keller NP, Hoffmeister D (2007) Accurate prediction of the *Aspergillus nidulans* terrequinone gene cluster boundaries using the transcriptional regulator LaeA. Fungal Genet Biol **44**: 1134–1145
- Endo A, Kuroda M and Tanzawa K (1976) Competitive inhibition of 3-hydroxy- 3- methylglutaryl coenzyme A reductase by ML-236A and ML-236B fungal metabolites, having hypocholesterolemic activity. FEBS Lett **72**: 323–326
- Fernandes M, Keller NP, Adams TH (1998) Sequence-specific binding by *Aspergillus nidulans* AflR, a C-6 zinc cluster protein regulating mycotoxin biosynthesis. Mol Microbiol **28**: 1355–1365
- Hamahata A, Takata Y, Gomi T, Fujioka M (1996) Probing the S-adenosylmethionine- binding site of rat guanidinoacetate methyltransferase. J Biochem **317** : 141–145
- Kale SP, Milde L, Trapp MK, Frisvad JC, Keller NP, J.W. Bok (2008) Requirement of LaeA for secondary metabolism and sclerotial production in *Aspergillus flavus*. Fungal Genet Biol **45**: 1422–1429
- Keller NP, Turner G, Bennett JW, (2005) Fungal secondary metabolism - from biochemistry to genomics. Nat Rev. Microbiol, **3**: 937-947
- Kosalkova K, Garcia-Estrada C, Ullan RV, Godio RP, Feltre R (2008) The global regulator LaeA controls penicillin biosynthesis, pigmentation and sporulation, but not roquefortine C synthesis in *Penicillium chrysogenum*. Biochimie **91**: 214–225
- Liu Y-G and Whittier RF (1995) Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. Genomics **25**: 674–681

- Park JW, Lee JK, Kwon TJ , Yi DH, Kim YJ (2003) Bioconversion of compactin into pravastatin by *Streptomyces* sp. *Biotechnol Lett* **25**: 1827–1831
- Perrin RM, Fedorova ND, Bok JW , Cramer RA, Wortman JR (2007) Transcriptional regulation of chemical diversity in *Aspergillus fumigatus* by LaeA. *Plos Pathog* **3**: 508–517
- Zerikly M, Challis GL(2009) Strategies for the discovery of new natural products by genome mining. *Chembiochem* **10** : 625–633.

Figure 1 Cloning of the *P. citrinum laeA* gene. (A) Amplification of the internal fragment of cDNA using degenerate primers D1 and D2. Lane I, The expected PCR fragment indicated by the arrow is 782 bp in size. M1, DNA Marker (2000 bp, 1500 bp, 1000 bp, 700 bp, 400 bp, 200 bp). (B) Amplification of *laeA* cDNA 3'-end by RACE with nested primers F1-3. The expected PCR fragment indicated by the arrow is 1136 bp in size. M2, DNA Marker (2000 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp). (C) Isolation of 5'-flanking region of *laeA* by TAIL-PCR using degenerate arbitrary primers AD1-4 and nested gene-specific primers R1-3. The expected PCR fragment indicated by the arrow is 981bp in size. No expected PCR product was obtained with the primers AD2/R1-3, AD3/R1-3 and AD4/R1-3. M2, DNA marker (2000 bp, 1000 bp, 750bp, 500 bp, 250 bp, 100 bp). (D) PCR amplification of the whole *Pci-laeA* genomic DNA and cDNA using primers LAF1 and LAR1. The *Pci-laeA* genomic DNA is of 1340 bp, and cDNA is of 1284 bp. M1, DNA Marker (2000 bp, 1500 bp, 1000 bp, 700 bp, 400 bp, 200 bp).