Journal of
Microbial & Biochemical Technology

Bhargavi et al., J Microb Biochem Technol 2015, 7:6
DOI: 10.4172/1948-5948.1000235

Research Article Open Access

Screening of Selected Soil and Endophytic Fungi for Lovostatin Biosynthetic Genes *lov*E and *lov*F

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Abstract

Lovastatin is a competitive inhibitor of the enzyme hydroxyl methylglutaryl coenzyme A reductase (HMGR) in cholesterol biosynthetic pathway and hence used in the treatment of hyperlipidemia. Our results revealed that *Aspergillus terreus* of soil origin produce copious amount of lovastatin than its counterpart that are endophytic in origin. Bioinformatics analysis of whole genome sequence of *A. terreus* (AH007774.1), a soil isolate revealed the presence of lovastatin gene cluster (AF141924.1 and AF141925.1), whereas, endophytic fungi including a species of *A. terreus* showed no homology with the lovastatin gene cluster. Molecular studies aimed at generating physical evidence were performed to analyze the expression of lovastatin biosynthetic genes *lov*E (regulatory gene) and *lov*F (transcriptional regulatory factor) in soil and endophytic fungi. The target PCR amplification of *lov*E (1512bp) and *lov*F (749bp) were successful in a strain of soil isolate, *Aspergillus terreus* (KM017693), whereas the same was not achieved in endophytic fungi. This is the first report on comparative analysis of complementary DNA sequence of a soil isolate and endophytic fungi, which further substantiate the absence of lovastatin production by endophytic fungi. The significance for the lack of lovastatin by endophytic fungi is also discussed.

Keywords: Lovastatin; *Lov*E; *Lov*F; *A. terreus*; Soil fungi; Endophytic fungi

Introduction

Lovastatin is a fungal secondary metabolite that competitively inhibits conversion of 3 hydroxy 3 methyl glutaryl coenzyme A (HMG CoA) to mevalonate in cholesterol biosynthesis. Since its discovery and approval by Food and Drug Administration (FDA), it has been prescribed worldwide as a potential anticholesterolemic agent [1]. Several fungi belonging to the genera of Aspergillus, Penicillium, Monascus, Pleurotus are reported to be lovastatin producers [2], however, *Aspergillus terreus* is the only fungal isolate utilized for commercial production of lovastatin [1].

The lovastatin gene cluster consists genes for lovastatin nonaketide synthase (*lov*B), lovastatin diketide synthase (*lov*F), enoyl reductase (*lov*C), transesterase (*lov*D), HMG-CoA reductase (ORF8), regulatory (*lov*E and ORF13) and cytochrome P450 monooxygenase. Lovastatin production is regulated by *lov*E and deletion of the same results in cessation of lovastatin production whereas gene duplication increases yield by 5-7 fold [3,4]. *Lov*F encodes a diketide synthase and is needed for attachment of 2 methyl butyric acid chain to monacolin J to form monacolin L [5].

The transcription rates of *lov*E and *lov*F have been studied in both submerged (SmF) and solid substrate fermentation (SSF) [6]. *Lov*F transcripts were detected from the day 1 of its growth period where as *lov*E was detected from day 3 onwards in SSF. In SmF transcription levels of both genes dropped as days progressed. *Lov*E genes isolated from *A. terreus* (CCTCC AF93208) was cloned into the vector pMD-19T (TaKaRa). Gene expression and sequencing studies confirmed the presence of *lov*E gene in transformants [3].

In view of the preliminary findings that endophytic fungi lack DNA sequences that are homologous to lovastatin biosynthetic pathway genes [7], the present study was undertaken to obtain further insights into the lovastatin biosynthetic pathway genes in both soil and endophytic isolates using the two widely studied genes *lov*E and *lov*F.

Materials and Methods

Isolation of endophytic fungi

Endophytic fungi from different plants were isolated and characterized as described previously [8].

Reinfection of plants with endophytic fungi

Selected medicinal plant seeds (*Piper nigrum, Garcinia cambogia, Coleus aromaticus, Venttivera zazonoides, Patchouli pogestomon*) were purchased from Dhanvanthrivana, Jnanabharathi campus, Bangalore University, Bangalore, India. Seeds were washed thrice with sterile distilled water and sterilized with 2% sodium hypochlorite for 5 min. Washed seeds were ger minated on Murashige-Skoog medium [9] supplemented with 2% sucrose in temperature-controlled (25°C) growth chambers under a 16 h light: 8 h darkness cycle for 2-3 weeks. Endophyte infection was performed by spraying spore suspension (10^6 spores/ml) of selected endophytic fungi (*Collectotrichum* sp., *Aspergillus terreus*, *Sordaria* sp., *Curvularia* sp., *Pithomyces* sp., *Pestalotiopsis* sp., *Phomopsis* sp., *Phoma* sp., *Podospora* sp., *Botrytis* sp.,) onto the leaves of each plantlet. Plantlets were allowed to grow in the greenhouse for 2 months and leaves were tested for the presence of the inoculated endophytic fungi [10].

Culturing of fungal strains

To 100 ml of Potato Dextrose Broth (PDB), 10µl of spore suspension

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Received September 21, 2015; **Accepted** October 01, 2015; **Published** October 08, 2015

Citation: Bhargavi SD, Praveen VK, Savitha J (2015) Screening of Selected Soil and Endophytic Fungi for Lovostatin Biosynthetic Genes *lov*E and *lov*F. J Microb Biochem Technol 7: 334-337. doi:10.4172/1948-5948.1000235

(10^7/10^8 spores/ml) of test organisms was inoculated. The flasks were kept on orbital shaker at 120 rpm at 30°C for 5 days followed by extraction of total RNA from fungal mycelia [8].

Extraction of total RNA

Total RNA from fungi was extracted following two different methods given below. Glassware used for total RNA isolation were treated with Di-Ethyl-Pyro-Carbonate (DEPC) water and dried overnight in the hot air oven.

TRIZOL method

One gram of filtered fungal biomass was ground to a fine powder under liquid N_2 followed by the addition of 1ml of Trizol reagent (Sigma T9424), incubated for 15 min and 0.2 ml of chloroform was added. Centrifugation was carried out at 12,000 rpm for 15 min at 4°C. Aqueous phase was transferred to a fresh tube and 0.5 ml of 2-propanol was added. Sample was allowed to stand for 5–10 min at room temperature followed by centrifugation at 12,000 rpm for 10 min at 4°C. Supernatant was decanted and 1 ml of absolute alcohol was added to the pellet. Centrifugation was carried out at 12,000 rpm for 15 min at 4°C. Supernatant was discarded and the pellet was washed with 70% alcohol and re-suspended in DEPC water for subsequent use [11].

STE (Sodium chloride, Tris and EDTA) method

One gram of filtered fungal biomass was homogenized under liquid nitrogen in a pre-cooled mortar, transferred to a tube containing 500 µl extraction buffer (0.1 M Tris-HCl (pH 8.0), 2 mM EDTA (pH 8.0),0.2 M NaCl, 1% SDS) and mixed gently. Equal volume of PCI (Phenol/Chloroform/Isoamyl alcohol (25:24:1, V/V/V)) was added immediately and mixed for 30 sec. The supernatant was pooled and PCI treatments were done twice to remove protein. Sodium acetate (1/10 volume) (Ph 5.5) and 2.5 volume ethanol were added and mixed well, kept at -20°C for 40 min. After centrifugation the pellets were washed with 70% ethanol. Finally, the pellets were air dried and suspended in 100µl DEPC-treated water [12]. Resultant total RNA was visualized on 1% agarose gel and used for the construction of c-DNA.

Construction of cDNA by reverse transcription

Isolated total RNA was treated with DNase for 10 min at 37°C to remove residual DNA, if any, and the RNA was used for cDNA construction by following the instruction of First Strand cDNA synthesis kit for RT-PCR (AMV) from Roche, Germany (Kit No. 11483188001). The reaction was initiated by adding 2 µl of 10X Reaction buffer, 4 µl of $MgCl_2$ (25 mM), 2 µlof deoxynucleotide mix, 2 µl of oligo-$P(dT)_{15}$ primer, 1 µl RNase inhibitor, 0.8 µl of reverse transcriptase enzyme. To the above mixture 4 µl of the isolated total RNA was added with variable volume of PCR grade water making up the volume to 20 µl, mixed well and briefly centrifuged to collect the reaction mixture at the bottom of the reaction tube. Reaction mixture was incubated at 25°C for 10 min and then 42°C for 60 min during which primer annealing and reverse transcription occurs respectively. Following 42°C incubation, the reverse transcriptase enzyme was denatured by incubating the reaction mixture at 99°C for 5 min and immediate cooling at 4°C for 5 min [13].

Amplification of *lov*E and *lov*F genes

Primer sequence for *Lov*E [3]

Forward: 5'-ATGGCTGCAGATCAAGGTATAT-3'

Reverse: 5'-TCATGGAGGAATATTGTTGAGG-3'

***Lov*F**

Forward: 5'-GCGTCGGTACATAAGGGGGG-3';

Reverse: 5'GTGGTTCCAAGGGTAGGGCGG-3'

Ribosomal 18S rRNA[6]

Forward: 5'-CGGCCCTTAAATAGCCCGGTCCG-3'

Reverse: 5'-CGAGCCATTCAATCAATCGGTAGTAGCG-3'

Primers for all targets were synthesized by Sigma (India) and Merck (India).The constructed cDNA was used as a template for PCR amplification using the designed primers. One Taq2X Master Mix with standard buffer (M0482S-NEB, England) was used for PCR amplification. This master mix was used at 1X concentration with DNA template and primers in a total reaction volume of 25 µl.Amplification conditions were set as follows: denaturation at 94°C for 5 min; then 40 cycles, each consisting of denaturation (94°C for 30 seconds), annealing (50°C for 20 seconds) and extension (72°C for 1 min and 40 seconds); followed by a single extension at 72°C for 20 min [14]. Amplified product size of *lov*E, *lov*F, and 18S ribosomal RNA was expected to be 1512 bp, 749 bp and 300 bp respectively. The amplified PCR products were run on 1% agarose gel and visualized for *lov*E, *lov*F and Ribosomal 18SrRNA amplicons with the molecular weight marker.

Results and Discussion

Lovastatin is widely used as an anticholesterolemic drug worldwide. Researchers have screened several microbes for lovastatin production from various niches [5]. Also, comparative studies on lovastatin production by SmF and SSF have been reported at molecular level in soil fungi. Lovastatin production which is chiefly regulated by *lov*E and *lov*F has been widely studied and gene transfer studies indicate a pivotal role of *lov*E in cross platform gene transfer [6].

Although, endophytic fungi are now being exploited for various pharmaceutically valuable secondary metabolites, there are a very few reports of lovastatin which is also a secondary metabolite being produced by endophytes. We have previously demonstrated that endophytic fungi as poor producers of lovastatin since none of the 54 endophytic fungi isolated by us from medicinal plants produced any detectable level of lovastatin even after 10 days of incubation [9]. Also, it was noticed that *Aspergillus terreus*, an endophyte, did not produce lovastatin while a soil isolate of the same fungus produced significant levels of lovastatin under identical growth conditions [8]. Since not much information is available on lovastatin production by endophytic fungi, this study was designed to analyze and compare the genetic makeup of lovastatin producing *Aspergillus terreus* (KM017963), a soil isolate, with selected endophytic fungi of medicinal plants.

The bioinformatics study of whole genome sequences of endophytic fungi and a soil fungus (obtained from NCBI GenBank) for the presence of lovastatin gene cluster (AF141924.1andAF141925.1) was carried out. Whole genome sequence of *A. terreus* (AH007774.1), a soil isolate exhibited the presence of lovastatin gene cluster showing 100% homology, whereas, all other endophytic fungi including a species of *Aspergillus terreus* showed no homology with the lovastatin gene cluster [7]. *Lov*E and *lov*F are the part of this gene cluster.

*Lov*E and *lov*F genes are involved in the lovastatin biosynthetic pathway. *Lov*E gene encodes a transcription factor which regulates the expression of genes that participate in the biosynthetic pathway, whereas *lov*F gene encodes a diketide synthase (DKS), one of the two polyketide synthases involved in lovastatin biosynthesis [6]. Thus both

*lov*E and *lov*F are two important genes in the biosynthetic pathway of lovastatin. Therefore, studies at the transcriptional level were undertaken to ascertain the lack of lovastatin expression as measured by the lack of *lov*E and *lov*F transcripts and the potential reason for the absence of *lov*E and *lov*F gene expression in endophytes.

The study was initiated by isolating total RNA from the lovastatin producing fungus, *A. terreus* (KM017693) and 10 endophytic fungi namely *Collectotrichum* sp., *A. terreus*, *Sordaria* sp., *Curvularia* sp., *Pithomyces* sp., *Pestalotiopsis* sp., *Phomopsis* sp., *Phoma* sp., *Podospora* sp., *Botrytis* sp., isolated by us from medicinal plants. Reinfection of plants with isolated endophytic fungi confirmed its endophytic nature. Trizol reagent yielded total RNA in *A. terreus* (KM017693) and endophytic fungi except in *Colletotrichum* sp. Therefore, STE buffer was used to extract total RNA from the *Colletotrichum* sp. Agarose gel electrophoresis was subsequently used to check the integrity of the total RNA. Agarose gel separation of ribosomal RNA including 18S rRNA and 28S rRNA subunits into distinct bands can be observed (Figure 1). Extraction of RNA was followed by construction of complementary DNA using reverse transcriptase enzyme. The resultant c-DNA was further used as a template for the amplification of desired genes (*lov*E and *lov*F).

Reverse transcription followed by PCR amplification yielded PCR products (amplicon) for *lov*E (1512 kb), *lovF* (749bp) and 18S Ribosomal RNA (300bp) with *Aspergillus terreus.*

Ribosomal 18S rRNA (300bp) was used as a housekeeping gene

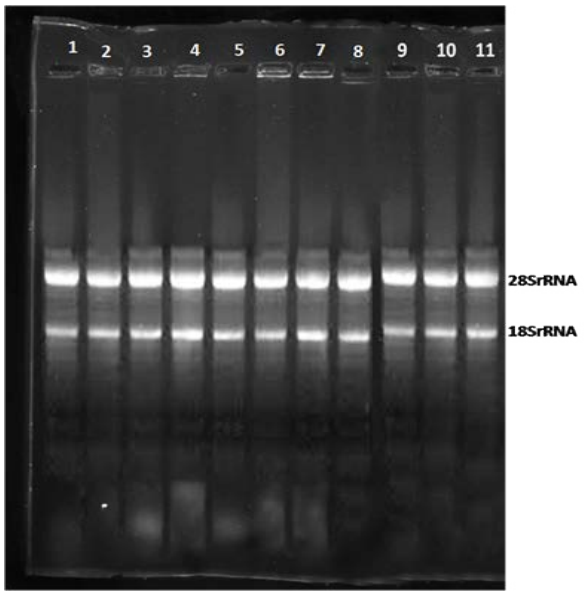

Figure 1: Total RNA of *Aspergillus terreus* and endophytic fungi on agarose gel.
Lane 1: *A. terreus* (KM017963),
Lanes 2 to 11: Total RNA isolated from endophytic fungi,
Lane 2: *Collectotrichum.* sp.,
Lane 3: *Aspergillus terreus*,
Lane 4: *Sordaria.* sp.,
Lane 5: *Curvularia.* sp.,
Lane 6: *Pithomyces.* sp.,
Lane 7: *Pestalotiopsis.* sp.,
Lane 8: *Phomopsis* sp.,
Lane 9: *Phoma.* sp.,
Lane 10: *Podopspora* sp.,
Lane 11: *Botrytis.* sp., respectively.

Figure 2: Electrophoresis of PCR products of *Aspergillus terreus* (L1) and endophytic fungi on agarose gel.
Lane 1 and 19: DNA marker (1500 bp to 100 bp)
Lane 2, 3 and 4: *Lov*E, 18SrRNA and *Lov*F amplicons of *Aspergillusterreus* (soil fungus, KM017963)
Lanes 6,9,12,15,18,22,25,28,31 and 34: PCR amplicons of 18S rRNA in endophytic fungi
Lanes 5,7,8,10,11,13,14,16,17,20,21,23,24,26,27,29,30,32,33,35: Absence of *Lov*E and *Lov*F PCR amplicons in endophytic fungi.

(control gene) for all the fungi used. The gene coding for 18S rRNA is a constitutive gene and expected to be present in all the samples. The presence of amplicon for 18S rRNA is indicative of the suitability of the assay conditions and test reagents used. Thus, it is also used as a validator of the results for the test organisms utilized in the study.

None of the ten endophytic fungi used in the study showed the presence of *lov*E or *lov*F amplicons but they showed the presence of 18S rRNA amplicon at about 300 bp. These results clearly indicate that endophytic fungi do not express lovastatin biosynthetic pathway genes that are homologous to *A. terreus lov*E or *lov*F (Figure 2).

Results of our previous biochemical [8] bioinformatics [7] and our present molecular level approach have categorically enabled us to conclude that most, if not all of the endophytic fungi may not be the potential candidates for lovastatin production. The absence of lovastatin production by endophytic fungi may be attributed to the loss of genes responsible for lovastatin production during co-evolution along with host plant [14,15]. In plant system, HMGR plays a vital role in the mevalonate pathway for isoprenoid biosynthesis for normal plant development and adaptation to environmental stress [16]. If lovastatin inhibits HMG CoA reductase (HMGR), subsequent synthesis of plant growth promoting substances and pigments such as ABA, gibberellins, ubiquinone, isoprenoids, chlorophylls and carotenoids get inhibited as mevalonate is the precursor for the synthesis of above compounds. Further, inhibition of the growth of tobacco cells was observed at low concentrations of lovastatin and restoration of such inhibition by cytokinin has also been reported [17-20]. However, as observed by Crowell and Salaz, reduction of HMG COA to mevalonate is not the only rate limiting step in cytokinin biosynthesis.

It was also reported that lovastatin inhibits the biosynthesis of α-Farnesene, a major ester formed during scald development and fruits ripening in 'golden supreme' apples [21].

Thus, in light of the harmful effects of the fungal metabolite lovastatin on plants, it is tempting to speculate that the lack of lovastatin production in endophytic fungi is not to harm the plant host system in which they are residing in. This is been proved by unexpressed lovastatin

biosynthetic genes *lov*E (regulatory gene) and *lov*F (transcriptional regulatory factor) in endophytic fungi in our present study. However, this result will further be validated by sequencing the whole genome of *Aspergillus terreus* and comparing with that of endophytic fungi for lovastatin gene cluster.

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