Process Biochemistry 47 (2012) 843–852

Contents lists available at SciVerse ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Is quorum sensing involved in lovastatin production in the filamentous fungus *Aspergillus terreus*?

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ARTICLE INFO

Article history:
Received 24 November 2011
Received in revised form 7 February 2012
Accepted 25 February 2012
Available online 3 March 2012

Keywords:
Fermentation
Quorum sensing
Butyrolactone
Lovastatin
Aspergillus terreus
Autoinducer

ABSTRACT

A novel role for butyrolactone I as a quorum sensing molecule in the filamentous fungus *Aspergillus terreus* is proposed based on its ability to affect both lovastatin and its own production.

In this work, the exogenous addition of 100 nM butyrolactone I at 96 h post-inoculation to submerged cultures of *A. terreus* in 5 L bioreactors resulted in 2.5-fold increase in lovastatin production as compared to control cultures at 168 h. An increase in endogenous butyrolactone I levels (2.5-fold) is also detected in cultures supplemented with butyrolactone I, thus suggesting an auto-stimulatory function. Molecular analysis of butyrolactone I-mediated enhancement of lovastatin production revealed induction of lovastatin biosynthetic genes, *lovB* and *lovF*, at the transcriptional level. Microarray analysis of *A. terreus* transcriptome depicted a growth phase-specific response to butyrolactone I addition as the majority of the expressed genes showed differential expression during the specific growth phase ($p < 0.01$). This study demonstrates for the first time the potential of butyrolactone I as a growth phase-specific inducer of the secondary metabolite lovastatin production and shows the auto-stimulatory effect of this molecule on its own production in the filamentous fungus *A. terreus*.

1. Introduction

Quorum sensing is an established phenomenon of microbial communication and regulation of gene expression, which has been extensively studied in both Gram-positive and Gram-negative bacteria for the last 40 years [1]. Quorum sensing in bacteria is mediated by small, diffusible molecules, produced by the microbial population, which accumulate throughout the microbial growth and act as "autoinducers", thereby activating a number of cell density related processes upon reaching a specific concentration [2]. This phenomenon was later discovered and characterised in the dimorphic fungus *Candida albicans*, where mycelia formation appears to be controlled by two different signals, namely farnesol and tyrosol [3,4].

Only recently, quorum sensing process has been reported in filamentous fungi.

Multicolic acid and related derivatives are proposed as quorum sensing molecules in the filamentous fungus *Penicillium sclerotiorum*, where they appear to be related to the production of the secondary metabolite sclerotiorin. In particular, the exogenous addition of an ethyl acetate extract of supernatants from *P. sclerotiorum* Strain M, which produces both γ-butyrolactone and sclerotiorin, to submerged cultures of the same strain resulted in a significant increase in sclerotiorin yield. However, the greatest enhancement in sclerotiorin production was obtained when spent medium containing γ-butyrolactone molecules from Strain M was added to cultures of *P. sclerotiorum* Strain S, a low sclerotiorin producing strain [5].

Filamentous fungi of the genus *Aspergillus* also appear to use cell–cell communication processes to regulate biomass-related functions, such as morphogenesis and secondary metabolite production. Oxylipins (linoleic acid-derived compounds) have been shown to induce the production of the secondary metabolite lovastatin in the ascomycete *Aspergillus terreus*. Exogenous addition of linoleic acid to low cell density cultures of *A. terreus* resulted in a significant increase in the transcription levels of lovastatin biosynthetic gene, thus confirming that oxylipins act as signalling molecules for this organism [6].

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1359-5113/$ – see front matter © 2012 Published by Elsevier Ltd.
doi:10.1016/j.procbio.2012.02.021

A. terreus has emerged as a pharmaceutically important fungus due to its ability to produce a wide variety of secondary metabolites, and predominantly the cholesterol-lowering drug lovastatin [7]. The inhibiting activity of lovastatin towards the hydroxymethylglutaryl coenzyme A reductase, which is the rate-limiting enzyme in cholesterol biosynthesis, has been exploited to reduce cholesterol levels in humans [8,9]. Lovastatin biosynthesis in *A. terreus*, is catalysed by two iterative Type I polyketide synthases, namely the nonaketide synthase LovB and the lovastatin diketide synthase LovF, as well as numerous accessory enzymes [10].

A. terreus is also a producer of γ-butyrolactone-containing molecules such as butyrolactone I and butyrolactone II [11]. In particular, butyrolactone I or α-oxo-β-(*p*-hydroxyphenyl)-γ-(*p*-hydroxy-*m*-3,3-dimethylallyl-benzyl)-γ-methoxycarbonyl-γ-butyrolactone has a major role in eukaryotic cells, as it acts as a potent inhibitor of cyclin-dependent kinases, whose function is to regulate cell cycle progression by controlling the phosphorylation and dephosphorylation cascade [12,13]. As γ-butyrolactone and its derivatives are well established signalling molecules in both Gram-positive and Gram-negative bacteria [14], and recently in the filamentous fungus *P. sclerotiorum*, this work aimed to determine whether butyrolactone I functions as a quorum sensing molecule in *A. terreus*. The effect of butyrolactone I on production of lovastatin in *A. terreus* has been previously investigated and the addition of butyrolactone I to *A. terreus* growing cultures was reported to cause enhancement in lovastatin yield, increased sporulation and changes in hyphal tip numbers [15]. However, the possibility that butyrolactone I might act as a quorum sensing signalling molecule in this filamentous fungus was not explored.

As quorum sensing molecules are known to affect secondary metabolite production by regulating the transcription of genes involved in their biosynthesis, the present study explored the effect of butyrolactone I on the expression of lovastatin biosynthesis genes at the transcriptional level. In addition, transcriptome-wide effects of butyrolactone I supplementation were also investigated to further elucidate its role in this fungus. Furthermore, butyrolactone I was found to enhance its own production, thereby suggesting butyrolactone I as a quorum sensing molecule in *A. terreus*.

2. Materials and methods

2.1. Strain and chemicals

A. terreus MUCL 38669 was used in this study for butyrolactone I and lovastatin production. This strain was obtained from CABI Biosciences UK Centre, Surrey, United Kingdom. All materials used in this study were obtained from Sigma–Aldrich Company Limited, Dorset, United Kingdom unless stated otherwise. All high-pressure liquid chromatographic (HPLC) assays were carried out using HPLC grade chemicals and water purchased from VWR International (Lutterworth, UK).

2.2. Media and growth conditions

2.2.1. Aspergillus terreus maintenance

A. terreus cultures were maintained on yeast and malt extract (YME) agar slopes, with the following composition: yeast extract (4.0 g l^{-1}); malt extract (10.0 g l^{-1}); glucose (4.0 g l^{-1}); agar (20.0 g l^{-1}). Freshly inoculated slants were incubated at 28 °C for 7 days for the development of spores and subsequently stored at 4 °C.

2.2.2. Shaken flask studies

Spores from the *A. terreus* slants were harvested using a sterile solution of 0.01% Tween 80 (v/v) supplemented with glass beads. The spores were counted using a haemocytometer. The spore suspension was inoculated into 100 ml growth medium to make up a final spore concentration of 10^7 ml^{-1}. The inoculated medium was incubated for 25 h at 27 °C on a rotary shaker at 220 rpm (2 cm throw). Ten milliliters of the culture was then transferred into 100 ml of GPY-L (25.0 g l^{-1} Glucose; 24.0 g l^{-1} Peptonised milk; 2.5 g l^{-1} Yeast extract; 50.0 g l^{-1} Lactose) production medium and incubated at 27 °C on a rotary shaker at 220 rpm for 216 h. Both growth and production media were prepared as previously described [15]. Samples were collected at 24 h intervals post-inoculation and processed for further analysis. The experiments were carried out three times, each time in triplicate.

2.2.3. Bioreactor studies

A 5 l Stirred Tank Reactor (5 l STR, FerMac360, Electrolab Ltd., UK) with an internal diameter of 16 cm, shaft length of 31.5 cm and two Rushton impellers was used for bioreactor studies. Bioreactors containing 3.5 l GPY-L production medium were autoclaved at 121 °C for 45 min. The reactor medium was inoculated by 10% (v/v) vegetative culture grown in shaken flask at 27 °C for 25 h. Two bioreactors were run simultaneously as control and test (100 nM butyrolactone added). The airflow rate and temperature were set at 1.0 vvm and 27 °C, respectively. The dissolved oxygen tension (% DOT), pH, and temperature were monitored throughout the fermentation. The stirrer speed was increased gradually from initial 150 rpm to 350 rpm during the course of fermentation to keep % DOT above 30. Samples were collected at 24 h intervals and assayed for lovastatin and butyrolactone I production, pH and carbohydrate utilisation.

2.2.4. Addition of butyrolactone I to lovastatin producing cultures

Butyrolactone I was obtained from BIOMOL International, UK. It was dissolved in ethanol and added to *A. terreus* test cultures to a final concentration of 100 nM (0.04 mg l^{-1}).

2.3. HPLC assays

2.3.1. Lovastatin assay

Lovastatin was quantified from methanolic extracts of *A. terreus* cell pellets by using reverse-phase chromatography on C18 column (Dionex, Acclaim® 120 (C18, 5 μM × 4.6 mm × 150 mm). Lovastatin extraction and quantification were carried out as previously described [6].

2.3.2. Butyrolactone I assay

For the quantification of butyrolactone I in *A. terreus* culture supernatants and cell pellets collected throughout fermentation, methanolic extraction was performed. Culture supernatants were separated from cell pellets by centrifuging the samples at 20 °C, 13,000 rpm for 20 min. An equal volume of methanol was added to the culture supernatant and incubated for 30 min at 220 rpm on a rotary shaker. Cell pellets were treated with methanol at a 1:1 ratio (w/v) and homogenised for 40 s at 4.5 m/s (meter/second) using a FastPrep-24 Instrument, (MP Biomedicals, Cambridge, UK). Butyrolactone I was quantified from the methanolic layer by Ultimate 3000 HPLC (Dionex, Camberley, UK) using a C18 column (Dionex, Acclaim® 120; C18, 5.0 μM × 4.6 mm × 150 mm). Mobile phase and flow rate used for butyrolactone detection are the same described for lovastatin. Butyrolactone I was detected at 308 nm and quantified using butyrolactone I standard (BIOMOL International, UK).

2.4. Molecular biology techniques

2.4.1. Total RNA isolation

Mycelia (0.1 g) were frozen using liquid nitrogen and ground using RNase free microcentrifuge pestle and mortar. The mycelia were resuspended in 450 μl of RLT (RNeasy Lysis Tissue) buffer (Qiagen, Crawley, UK) by vortexing vigorously and incubated at 56 °C for 2 min. To aid further cell lysis the mix was passed 5 times through a 0.5 μm syringe needle. RNA isolation was carried out using the RNeasy Plant mini kit from Qiagen as instructed by the manufacturer. DNA contamination was eliminated by treatment with DNase (Qiagen). The integrity of the extracted RNA was confirmed by gel electrophoresis analysis using a formaldehyde-treated 1.2% agarose gel. Total RNA concentration was determined by recording A_{260} with a Perkin Elmer Lambda 35 UV/VIS spectrophotometer (Cambridge, UK) and the samples were stored at −80 °C for future use.

2.4.2. Complementary DNA preparation (cDNA)

Complementary DNA from each sample was obtained by reverse transcription performed with 100 ng of total RNA using the ImProm II Reverse Transcription System (Promega, Southampton, UK). The reaction was carried out using the oligo d(T) primers provided and by following the manufacturer's protocol. The resulting cDNA was stored at −20 °C for future use.

2.4.3. Primer design for conventional PCR

Primers for conventional and real time quantitative PCR (qPCR) for *A. terreus* MUCL 38669 were designed using Primer 3 software. The primer sequences are shown in Table 1. Primers were designed for the ketosynthase (KS) region of *lovB* (lovastatin nonaketide synthase) and *lovF* (lovastatin diketide synthase). Primers were custom prepared by Invitrogen (Renfrew, UK).

2.4.4. Real time quantitative PCR (qPCR)

Real-time PCR was carried out using primers for the KS regions of *lovB* and *lovF* for the control as well as the test samples. Gene quantification was performed using the ABI Prism 7000 Sequence Detection system (Applied Biosystems, Warrington, UK) with 96-well MicroAmp optical plates (Applied Biosystems). SYBR Green Two-step kit from Sigma–Aldrich was used as a detector for the real-time PCRs. Reaction mixtures comprised SYBR Green jump start *Taq* Ready mix (12.5 μl), ROX internal reference dye (0.5 μl), cDNA template (2.5 μl), forward and reverse primers (2.5 μl each). DMSO (0.25 μl) and glycerol (2 μl) were also added to the reaction mix to aim