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Reactive oxygen species regulate lovastatin biosynthesis in *Aspergillus terreus* during submerged and solid-state fermentations

Roxana U. MIRANDA[a,b], *Luis E. GÓMEZ-QUIROZ*[b], *Mariel MENDOZA*[a], *Ailed PÉREZ-SÁNCHEZ*[a], *Francisco FIERRO*[a], *Javier BARRIOS-GONZÁLEZ*[a,*]

[a]*Departamento de Biotecnología, Universidad Autónoma Metropolitana –Iztapalapa, Av. San Rafael Atlixco 186, Col. Vicentina, Iztapalapa, 09340 México D.F., Mexico*

[b]*Departamento de Ciencias de la Salud, Universidad Autónoma Metropolitana –Iztapalapa, Av. San Rafael Atlixco 186, Col. Vicentina, Iztapalapa, 09340 México D.F., Mexico*

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ABSTRACT

In a previous work we detected an important increase in reactive oxygen species (ROS) concentrations during idiophase in lovastatin fermentations. Hence, the objective of the present work was to determine if ROS contributes to the regulation of lovastatin biosynthesis. Exogenous antioxidants were used to reduce ROS accumulation. The addition of N-Acetyl-L-cysteine (NAC) decreased ROS accumulation and concurrent lovastatin production. In solid-state fermentation (SSF), the addition of 100 mM of NAC lowered ROS accumulation by 53 %, together with a 79 % decrease in lovastatin biosynthesis. A similarly, situation was observed in submerged fermentation (SmF). Decreased lovastatin production was due to a lower expression of the regulatory gene *lovE*, and gene *lovF*. Moreover, the addition of H_2O_2 to the culture caused precocious gene expression and lovastatin biosynthesis. These results indicate that ROS accumulation in idiophase contributes to the regulation of the biosynthetic genes. It was considered that Yap1 (Atyap1) could be a transcription factor linking ROS with lovastatin biosynthesis. In a Northern analysis, *Aspergillus terreus yap1* gene (Aty*ap1*) was highly expressed during trophophase but down regulated during idiophase. Conversely, expression pattern of *srrA* gene, suggested that SrrA could positively control lovastatin biosynthesis, and also explaining the characteristics of the biosynthesis in SSF.

Introduction

In filamentous fungi, secondary metabolites are often produced after the phase of rapid growth (trophophase), during a subsequent production stage or idiophase. In this case, secondary metabolism starts when growth is limited by the exhaustion of one key nutrient: carbon (glucose), nitrogen, or phosphate source, initiating a stage with low or nil growth rate, but high production rate (Barrios-González *et al.* 2005).

Modular biosynthesis of secondary metabolites uses monomeric units from primary metabolism as precursors (Gunnarsson *et al.* 2004). Their gene functions are governed

* *Corresponding author*. Tel.: +52 (55) 5804 4600x2871; fax: +52 (55) 5804 4712.
E-mail address: jbg@xanum.uam.mx (J. Barrios-González).
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by complex regulatory mechanisms, such as: induction by different environmental stimuli, carbon catabolite regulation, feedback regulation, and others (Brakhage 1998; Brakhage et al. 2009). Hence, industrial production media are designed to circumvent negative regulatory mechanisms, and to enhance positive ones (Sanchez & Demain 2002).

Certain broad domain transcription factors mediate some of the above mentioned regulatory mechanisms, like AreA (nitrogen regulation; Marzluf 1997) CreA (carbon catabolite repressor; Espeso & Peñalva 1992). In addition, many secondary metabolites, including lovastatin, are regulated through the global regulator of secondary metabolism LaeA (Bok & Keller 2004).

Genes encoding the enzymes of a secondary metabolite pathway are clustered in the genome and often include a regulator gene, encoding a cluster specific transcription factor. Several such transcription factors have been found which regulate secondary metabolite gene clusters, for instance LovE, regulating lovastatin biosynthetic genes in *Aspergillus terreus* (Kennedy et al. 1999), and AflR, regulating aflatoxin (AT), and sterigmatocystin biosynthetic genes in *Aspergillus parasiticus* and *Aspergillus nidulans* (Fernandes et al. 1998; Chang et al. 1999).

Lovastatin is a commercially valuable secondary metabolite produced by *A. terreus* and *Monascus purpureus*, although the former is used for industrial production. This compound, as well as its immediate derivative simvastatin, lower cholesterol levels in blood (Endo 1992 2004; Barrios-González & Miranda 2010).

The lovastatin biosynthetic gene cluster consists of 18 putative open reading frames (ORFs), among which *lovE* was annotated to encode a regulatory protein. The *lovE* gene encodes a Zn2Cys6 type transcription factor (TF), and it is assumed to regulate lovastatin production at the transcriptional level. The biosynthesis cluster includes two type I polyketide synthase genes: *lovB* and *lovF*. The latter encodes the lovastatin diketide synthase, enzyme that specifies formation of 2-methylbutyrate and interacts closely with an additional transesterase (LovD) responsible for assembling lovastatin from this polyketide and monacolin J (Kennedy et al. 1999).

Despite the knowledge of the genes and the enzymes involved in the biosynthetic pathway, little effort has been directed towards studying the regulation and the physiology of lovastatin biosynthesis. However, from production medium optimization studies, it is evident that lovastatin is carbon catabolite regulated, probably mediated by CreAp (Hajjaj et al. 2001). Hence, the onset of lovastatin biosynthesis, after glucose exhaustion, can be attributed to relief from carbon catabolite repression, and the change to lactose consumption during idiophase. In addition, some evidence indicates that lovastatin biosynthesis in *A. terreus* undergoes negative feedback regulation i.e., lovastatin inhibits its own biosynthesis (Jia et al. 2010).

However, studies on lovastatin biosynthesis in solid-state fermentation (SSF) have revealed the existence of other as yet uncharacterized factors or stimuli that influence the expression of lovastatin biosynthetic genes. In SSF, secondary metabolites are often produced in higher yields as compared to submerged fermentation (SmF), and it is thought to be due to the different physiology displayed by the fungus in SSF (Barrios-González 2012).

Lovastatin specific production, during SSF on polyurethane, has been measured to be 14 times higher than that obtained in SmF (Baños et al. 2009). In addition, SSFs systems employing this artificial inert support permit the use of absorbed liquid culture media, making it a clean and comparable system that facilitates basic studies (Barrios-González et al. 2008).

Searching for the environmental stimuli specific for SSF which induce this higher lovastatin production, we have recently found that direct contact with air is a very important stimulus inducing the higher production, and considered that its stimulating effect could be through oxidative stress or reactive oxygen species (ROS) formation (manuscript in preparation).

Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet}$), consist of radical and non-radical oxygen ROS, and are generated as metabolic by-products of aerobic growth. An excess of ROS leads to oxidative stress by directly or indirectly damaging DNA, proteins, and lipids (Gutteridge & Halliwell 1999). In contrast, evidence is accumulating that ROS also provide vital signalling functions for diverse cellular processes (Rhee 2006; Veal et al. 2007). In recent years, many authors have found evidence of a close association between ROS and development and differentiation in fungi, for example increased ROS levels have also been detected during cell differentiation in *A. nidulans* (Aguirre et al. 2005; 2006).

In *A. parasiticus*, ROS accumulated during AT production phase (idiophase) (Jayashree & Subramanyam 2000; Narasaiah et al. 2006), indicating a link between ROS and AT biosynthesis.

Since ROS are constantly being generated, all aerobically growing cells maintain an assemblage of biochemical antioxidants and enzymes that facilitate the breakdown of ROS and keep the cell in a state of redox balance. Antioxidant defenses include superoxide dismutase, catalase, and glutathione peroxidase.

The signalling pathways that participate in response to oxidative stress were comprehensively dissected in yeast and were found to be conserved in filamentous fungi. These conserved signalling pathways incorporate a multistep phosphorelay system module, which relays the signal to the stress activated protein kinase/mitogen-activated protein kinase (SAPK/MAPK) pathway module, and this modulates activity of an array of specific oxidative/osmotic stress related TFs (Hong et al. 2013a; Miskei et al. 2009; Toone et al. 2001; Ikner & Shiozaki 2005; Toone & Jones 1998; Bahn et al. 2007).

It is considered that some oxidative stress-response TFs, associated to SAPK/MAPK cascade, like Yap1, AtfB, and SrrA, together with MsnA (linked to the cAMP-PKA signalling pathway), also contribute to the regulation of AT biosynthesis in *A. parasiticus*.

Yap1 is a bZIP TF in yeast and filamentous fungi that is activated in response to oxidative stress (Asano et al. 2007). Deletion of Apy*apA* (orthologue of Yap1 in *A. parasiticus*) resulted in an increased susceptibility to extracellular oxidants, precocious ROS, and AT accumulation and premature conidia formation as compared to the wild type strain (Reverberi et al. 2008), suggesting its participation in AT regulation by ROS.

In *Saccharomyces cerevisiae* SAPK/MAPK transmits osmotic stress signals through two response regulators: Ssk1 and Skn7, although oxidative stress appears to activate Skn7