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Sequencing, physical organization and kinetic expression of the patulin biosynthetic gene cluster from *Penicillium expansum*

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ABSTRACT

Patulin is a polyketide-derived mycotoxin produced by numerous filamentous fungi. Among them, *Penicillium expansum* is by far the most problematic species. This fungus is a destructive phytopathogen capable of growing on fruit, provoking the blue mold decay of apples and producing significant amounts of patulin. The biosynthetic pathway of this mycotoxin is chemically well-characterized, but its genetic bases remain largely unknown with only few characterized genes in less economic relevant species. The present study consisted of the identification and positional organization of the patulin gene cluster in *P. expansum* strain NRRL 35695. Several amplification reactions were performed with degenerative primers that were designed based on sequences from the orthologous genes available in other species. An improved genome Walking approach was used in order to sequence the remaining adjacent genes of the cluster. RACE-PCR was also carried out from mRNAs to determine the start and stop codons of the coding sequences. The patulin gene cluster in *P. expansum* consists of 15 genes in the following order: *patH*, *patG*, *patF*, *patE*, *patD*, *patC*, *patB*, *patA*, *patM*, *patN*, *patO*, *patL*, *patI*, *patJ*, and *patK*. These genes share 60–70% of identity with orthologous genes grouped differently, within a putative patulin cluster described in a non-producing strain of *Aspergillus clavatus*. The kinetics of patulin cluster genes expression was studied under patulin-permissive conditions (natural apple-based medium) and patulin-restrictive conditions (Eagle's minimal essential medium), and demonstrated a significant association between gene expression and patulin production. In conclusion, the sequence of the patulin cluster in *P. expansum* constitutes a key step for a better understanding of the mechanisms leading to patulin production in this fungus. It will allow the role of each gene to be elucidated, and help to define strategies to reduce patulin production in apple-based products.

1. Introduction

Patulin is a polyketide-derived mycotoxin, the risk of exposure to which has emerged as an important topic given the high number of toxic effects that have assigned to it. Patulin produces acute and chronic toxicity mainly genotoxicity, cytotoxicity, mutagenicity as well as immunotoxicity (Puel et al., 2010). It is currently one of the few mycotoxins whose levels in food are regulated in many countries around the world because of its perceived harmful effects in humans, particularly children, who are most susceptible to patulin toxicity (Brandon et al., 2012).

Patulin is produced by a number of species belonging to the *Aspergillus*, *Byssochlamys* and *Penicillium* genera (Varga et al., 2007; Houbraken et al., 2006). Of these, *Penicillium* is the most prolific with 14 species identified as patulin producers (Frisvad et al., 2004; Vansteelandt et al., 2012). Within this genus, *Penicillium expansum* is considered as the main source of patulin in food and consequently the species of greatest concern regarding public health and economic risk (Morales et al., 2008). *P. expansum* is the causal agent of a common post-harvest disease known as blue mold rot (Baert et al., 2007). Fruit products in general and apples in particular, are one of the sectors most affected by this pathogen, and are considered by far the main route of entry of patulin into the food chain (Chen et al., 2004; Baert et al., 2012). The European Union has set maximum acceptable levels of 50, 25 and 10 μg of patulin/kg, for fruit juices, nectars and fermented apple beverages, and solid apple products and apple-based products for infants and young children, respectively (EU Commission Regulation, 2003, 2006).

The understanding of the biosynthetic mechanisms of the most agriculturally important mycotoxins has greatly increased over the past

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decades. Nowadays, biosynthetic gene clusters have been identified for the major mycotoxins. The aflatoxin biosynthetic pathway and its 25 related genes have been entirely characterized in the most problematic aflatoxigenic fungi, *Aspergillus flavus* and *Aspergillus parasiticus* (Yu et al., 2004). The cluster of sterigmatocystin in *Aspergillus nidulans*, an intermediate in aflatoxin formation, has been shown to share 16 genes with the aflatoxin gene cluster but arranged differently (Brown et al., 1996). A considerable achievement was the characterization of the trichothecenes gene cluster in *Fusarium graminearum* (Kimura et al., 2003). A part of the ochratoxin A gene cluster was also characterized in the ochratoxigenic species *Penicillium nordicum* (Karolewiez and Geisen, 2005) and the zearalenone biosynthetic gene cluster of *F. graminearum* has been reported to contain four genes located within a 39 kb region (Lee et al., 2011). Recently, the fumonisins biosynthetic gene cluster was identified, with currently 16 genes involved in the biosynthesis of fumonisins in *Fusarium verticillioides* (Bojja et al., 2004; Sagaram and Shim, 2006; Proctor et al., 2013).

In the case of patulin, its biosynthetic pathway consists of about 10 enzymatic reactions as suggested by several biochemical studies and by identification of several mutants that are blocked at various steps in the biosynthetic pathway (Moake et al., 2005). Based on the sequence of the patulin non-producing strain of *Aspergillus clavatus* NRRL1, our team elaborated a putative gene cluster for the biosynthesis on this mycotoxin (Artigot et al., 2009). Out of the 15 genes, only five have been identified and characterized in *Penicillium griseofulvum*, *Byssochlamys nivea* or *A. clavatus*: *patK*, *patG*, *patH*, *patI*, and *patN* encoding respectively a 6-methylsalicylic acid synthase (Beck et al., 1990; Puel et al., 2007), 6-methylsalicylic acid decarboxylase (Snini et al., 2014), *m*-cresol hydroxylase (Artigot et al., 2009), *m*-hydroxybenzyl alcohol hydroxylase (Artigot et al., 2009) and isoepoxydon dehydrogenase (Fedeshko, 1992; Dombrink-kurtzman and Engberg, 2006; Puel et al., 2007).

A better understanding of mechanisms leading to patulin production in *P. expansum* will help to define strategies to reduce its presence in apple based products. Because *A. clavatus* does not grow on pomaceous fruits, it is necessary to obtain the sequence of the patulin cluster in *P. expansum*, the causal agent of blue mold rot, to study the regulatory mechanisms of patulin production in apples.

In this paper, we describe the identification, isolation and analysis of the patulin biosynthetic gene cluster from *P. expansum*. We also performed an analysis of the expression of the different genes of the cluster under both permissive and restrictive conditions for patulin production.

2. Materials and methods

2.1. Fungal growth and DNA isolation

A filamentous fungal strain of *P. expansum* NRRL 35695 isolated from grapes in the Languedoc Roussillon region of France was used in this study. The isolate was previously shown to belong to this *Penicillium* species according to the DNA sequencing of the ITS gene region. Furthermore, its ability to produce patulin was confirmed. The fungal strain was freshly cultivated on PDA media (Fluka, Saint-Quentin Fallavier, France) and allowed to grow for 7 days at 25 °C to enhance sporulation. This culture was then used to inoculate a 250 mL Erlenmeyer flask containing 100 mL of yeast extract-sucrose (YES) broth with 10^5 spores and it was then incubated at room temperature under constant agitation for 4 days. The liquid culture was filtered through Whatman No. 42 filter paper and the mycelium was ground up under liquid nitrogen. DNA was extracted according to the method described by Gardes and Bruns (1993) with some modifications. In this method, the powdered mycelium was collected in microfuge tubes, homogenized with 700 μL of lysis buffer [2% CTAB (Fluka, Saint-Quentin Fallavier, France), 1.4 M NaCl (Euromedex, Souffelweyersheim, France), 20 mM EDTA pH 8 (Sigma-Aldrich, Saint-Quentin Fallavier, France), 100 mM Tris–HCl pH 8 (Euromedex, Souffelweyersheim, France)] and incubated at 50 °C for 10 min then cooled on ice for 1 h. The sample was later treated with 500 μL of Phenol:Chloroform (50%/50%; v/v) (MP Biomedicals, IIIkirch, France; Sigma-Aldrich, Saint-Quentin Fallavier, France), vortexed for one minute and the supernatant collected after centrifugation at 16,200 ×*g* for 15 min at 4 °C. The DNA was precipitated with an equal volume of ice-cold isopropanol (Carlo Erba, Val-de-Reuil, France) and incubated overnight at −20 °C. After incubation, sample was again centrifuged at 16,200 ×*g* for 10 min at 4 °C. The pellet obtained was rinsed with 70% ethanol (Sigma-Aldrich, Saint-Quentin Fallavier, France), thoroughly air-dried and resuspended in 50 μL of sterile water. The DNA purity ratio and concentration were then measured using a NanoDrop ND1000 (Labtech, Palaiseau, France).

2.2. Strategy adopted to identify the patulin biosynthesis genes in *P. expansum*

2.2.1. Design of PCR primers

Several orthologous-gene alignments were carried out for almost all the genes of the patulin biosynthetic pathway previously identified in *A. clavatus*, *P. griseofulvum* and *Penicillium chrysogenum*. The exception was for the two genes *patE* and *patF* that have not been identified so far in a species of *Penicillium* and for *patM*, the gene encoding for an ABC transporter that had been sequenced in a previous study (Puel, 2007). The alignments were conducted using the MultAlin Multiple sequence alignment tool (http://multalin.toulouse.inra.fr/multalin/), and they have served as a highly useful resource for the design of PCR primers. The strains used and the sequence accession numbers are listed in Table S1. Degenerative PCR primers containing related sequences with differences at specific locations were designed based on the highly-conserved regions in these alignments to amplify overlapping fragments.

However, to amplify the intergenic regions, the hypothetical gene orders and orientations were assumed relying on the patulin biosynthesis gene homolog clusters in other fungal species, mainly *A. clavatus* for which the total cluster had been sequenced and *P. chrysogenum*, a non-producer species which is phylogenetically very close to *P. expansum*. The lengths of these intergenic regions were retrieved from the GenBank data and several gene-specific primer sets were designed to target these regions on the basis of the partial sequences determined during the course of this study. The primers were chosen to share an optimum theoretical melting temperature (Tm) of approximately 60 °C using the Oligo Calculator tool (http://mcb.berkeley.edu/labs/krantz/tools/oligocalc.html). All the primers used were synthesized by Sigma-Aldrich and their details are given in Table S2a.

2.2.2. PCR amplification

All the PCR amplifications were carried out in 50 μL reaction mixtures containing 5 μL of 10x PCR buffer, 1.5 μL of 50 mM $MgCl_2$, 4 μL of dNTP (2.5 mM each), 1.5 μL of each primer (at 0.1 μg/μL), 2.5 U of recombinant Taq DNA Polymerase (Invitrogen, Life Technologies SAS, Saint Aubin, France) and H_2O up to 50 μL. Three μg of genomic DNA was used as template. The PCR thermal cycling conditions were carried out in a thermocycler from Applied biosystems (GeneAmp PCR System 2700) as follows: a 5 min preheating step at 94 °C, followed by 40 cycles of amplification (94 °C for 45 s, 60 °C for 45 s, 72 °C for X s) and a final 10 min elongation step at 72 °C. The extension time (X) was selected according to the expected amplicon length.

2.2.3. Genome walking approach

The nucleotide sequences adjacent to known regions were determined according to an improved genome walking method, using the GenomeWalker Universal Kit from Clontech (Saint-Germain en Laye, France) as described by Dombrink-Kurtzman and Engberg (2006). For each walk, two gene specific primers were designed; one for the primary PCR (GSP1) and the other for secondary PCR (GSP2) on the basis of the newly identified gene fragments. The outward gene-specific primers