Characterization of a cytochrome P450 monooxygenase gene involved in the biosynthesis of geosmin in *Penicillium expansum*

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Geosmin is a terpenoid, an earthy-smelling substance associated with off-flavors in water and wine. The biosynthesis of geosmin is well characterized in bacteria, but little is known about its production in eukaryotes, especially in filamentous fungi. The origin of geosmin in grapevine is largely attributable to the presence of *Penicillium expansum* on grapes. Herein, we describe the characterization of “gpe1”, a gene encoding a cytochrome P450 monooxygenase probably involved in the biosynthesis of geosmin in this species. A gpe1 knockout mutant of *P. expansum* M2230 lost the capacity to produce geosmin, while the genetically complemented mutant restored it. The deduced gpe1 protein sequence shows identities with other cytochrome P450 monooxygenases involved in diterpene biosynthesis. These enzymes catalyze the addition of hydroxyl groups to the diterpene compounds. gpe1 protein could work in the same way, with sesquiterpenes as substrates. This gene seems to be only present in geosmin-producing *Penicillium* species. To our knowledge, this is the first characterization of a fungal gene encoding an enzyme involved in geosmin biosynthesis.

Key words: *Penicillium expansum*, cytochrome P450 monooxygenase, geosmin.

INTRODUCTION

Geosmin (trans-1,10-dimethyl-trans-9-decalol) is a small volatile isoprenoid compound responsible for an earthy-smelling off-flavor in water and foodstuffs, often associated with 2-methylisoborneol (Gerber and Lechevalier, 1965; Buttery and Garibaldi, 1976). It can be produced by many microorganisms, including actinomycetes, cyanobacteria, myxobacteria, several filamentous fungi, and may also be directly synthesized by liverworts, red beet, and insects (Izaguirre et al., 1982; Mattheis and Roberts, 1992; Omura et al., 2002; Spiteller et al., 2002; Lu et al., 2003; Dickschat et al., 2004; Zaitlin and Watson, 2006). Geosmin has a very low odor threshold, and numerous analysis methods are available (Cortada et al., 2011). Geosmin is notably found in drinking water and in grape juice. In the case of water, contamination is strictly bacterial (Jüttner and Watson, 2007), and physical, chemical and biological treatments exist (Cook et al., 2001; Kutschera et al., 2009; Eaton and Sandusky, 2010). In the case of wine, origin of geosmin is mainly due to the development of *Penicillium expansum* on grapes, with a possible impact of *Botrytis cinerea* (La Guerche et al., 2004; Morales-Valle et al., 2011). Removal or degradation processes will be detrimental to the organoleptic quality of wines, and...
two genes and those from *P. expansum*, suggesting another role for *p450-1* and *p450-2*. The latter showed higher similarities (40% on average) with cytochrome P450 involved in terpene metabolism and lower (less than 30%) with those involved in polyketide metabolism (as patulin for example).

In this study, we report the characterization of a P450 gene (*gpe1*) required for the geosmin biosynthesis in *P. expansum*.

**MATERIALS AND METHODS**

**Fungal strain and culture conditions**

*P. expansum* M2230 strain was grown for sporulation at 28°C on Yeast Extract Sucrose (YES) Agar medium (Yeast extract, 20 g; Sucrose, 150 g; Agar, 20 g; Distilled water, 1 L) for 7 days. Spores were collected using a solution of 0.01% (v/v) Tween 80, counted and stored at -20°C in 25% (v/v) glycerol before use. Conidia were inoculated (density ~ 10^6/ml) into 250 mL Erlenmeyer flasks containing 100 mL YES broth medium, and incubated at 28°C for 4 days, without shaking. Mycelium was harvested by filtration through a 0.45 µm filter, ground in liquid nitrogen and then stored at -80°C before nucleic acid extraction.

**DNA extraction and purification**

Extraction of genomic fungal DNA was done by a rapid extraction method (Liu et al., 2000). The extraction of DNA from plasmids was done by using a Pure Link Plasmid Miniprep Kit (Invitrogen, France). The PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, France). The quality and quantity of DNA were estimated by measuring optical density (OD), that is, OD 260 nm / OD 280 nm.

**PCR amplifications**

PCR amplifications were performed in 25 µL reaction mixtures containing 2.5 µL of *Taq* polymerase 10 X buffer with MgCl2, 0.5 µL of dNTPs mix 10 mM each, 0.5 µL of each primer 10 mM, 1 U of *Taq* polymerase (MP Biomedicals, France), ~ 200 ng of genomic DNA, sterile deionized H2O up to 25 µL. Reaction conditions were: 94°C for 4 min (initial denaturation), 30 cycles at 94°C for 45 s (denaturation), 2-5 degrees Celsius below the Tm of both primers for 45 s (annealing), and 72 °C for 1 min (elongation). A final elongation for 10 min at 72°C was added.

**Disruption of *gpe1* P450 gene in *P. expansum* M2230**

The disruption of *gpe1* was done by inserting the *E. coli* hygromycin B phosphotransferase gene (*hph*) flanked by *A. nidulans trpC* promoter and terminator sequences from plasmid pD2.1, as previously described by Bacha et al. (2009) and as illustrated in Figure 2. After construction of the transformation vector (Figure 2a), *gpe1* inactivation was achieved by transformation of *P. expansum* M2230 protoplasts with TopoPh (Figure 2b). Complementary mutants were obtained by transformation of Δ*gpe1* protoplasts with TopoP (Figure 2c). 40 mg/mL lysing enzymes (Sigma, France) were used for the preparation of protoplasts.
Figure 2. Schematic representation of transformation vector formation and gpe1 gene disruption. (a) Using primer pair mhsF/mhsR (Table 1), 1182 bp gpe1 gene containing SmaI restriction site (indicated by triangle) was amplified. PCR product was cloned into PCR2.1–Topo plasmid to generate plasmid TopoP. PID2.1 plasmid vector was restricted with PmlI (indicated by triangle) to obtain hph cassette (1032 bp). TopoP was restricted with SmaI and ligated with hph cassette to generate TopoP_hph transformation vector. (b) Protoplasts of P. expansum (wt) were prepared and gpe1 gene was disrupted using TopoP_hph vector to obtain Δgpe1 mutant. (c) Protoplasts of Δgpe1 mutant were prepared and gpe1 gene was restored using TopoP vector to obtain gpe1 complementary mutant.

Screening of the transformants

Hygromycin-resistant transformants were selected on YES medium (20 g/L of yeast extract, 1 M sucrose, 15 g/L of agar) supplemented with 150 µg/mL of hygromycin B. Transformant plates were incubated at room temperature for 24 h and then transferred to 28°C for 4 days. Hygromycin resistant transformants were further screened through a PCR, using hph gene specific primers hphF and hphR (Table 1). Positive transformants were then subjected to a second PCR using P450 gene specific primer mhsF with hphR. To screen the genetically complemented mutants, each of the colonies grown after 48 h of incubation was divided into two parts. One part was transferred to a Petri dish containing YES medium without hygromycin and the other part to another Petri dish containing YES medium with hygromycin (final concentration of 150 µg/mL). The colonies which grew successfully on YES medium without hygromycin but not on YES medium with hygromycin were subjected to different PCRs (as described above in case of mutants) for further screening.

Quantification of geosmin production

The production of geosmin was quantified from 10 days old culture of P. expansum wild type, Δgpe1 mutant and gpe1 complementary mutant strains grown in Petri dishes containing YES medium. We put all the mycelium along with medium in a tube after cutting it into small pieces with a sterile surgical blade. 10 mL of 20% ethanol were added in each tube containing all the mycelium of relevant strain. After vortexing, the tubes were incubated at room temperature at 200 rpm for 1 h. Then, filtered samples were sent to Exact Laboratory at Macon (France) for quantification of geosmin production, done by gas chromatography-mass spectrometry (GC-MS), with a limit of quantification of 10 ng/L.

Data analysis

The deduced amino acid sequence was determined using the http://www.expasy.org/tools/dna.html site while protein–protein Blast (Blastp) searches were conducted at the GenBank database http://www.ncbi.nlm.nih.gov. The alignments were conducted using the website http://multalin.toulouse.inra.fr/multalin. The sequence obtained was deposited in Genbank under the accession number JN126314.

RESULTS AND DISCUSSION

Considering that P. expansum also produce geosmin, and that this molecule belongs to the terpene family, so what about the involvement of p450-1 and p450-2 in geosmin biosynthesis? Moreover, these two partial sequences seemed to match with different parts of the same protein.

For this two primers were designed, mhsF corresponding to the 5' end of p450-2 and mhsR corresponding to the 3' end of p450-1 (Table 1). This allowed the amplification and the sequencing of a single 1182 bp P. expansum (strain M2230) gene fragment. The corresponding amino acid sequence (394 residues)
Table 1. PCR primers used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5'–3')</th>
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</thead>
<tbody>
<tr>
<td>mhsF</td>
<td>CGAATTTGCTGGAAAGCCG</td>
</tr>
<tr>
<td>mhsR</td>
<td>ATTTGCTTTCCGGTTCAGC</td>
</tr>
<tr>
<td>hphF</td>
<td>GAATTCAGCGAGAGGCCTGAC</td>
</tr>
<tr>
<td>hphR</td>
<td>ACATTTGGAGCGGAATTC</td>
</tr>
</tbody>
</table>

Figure 3. Alignment of the deduced amino acid sequence of gpe1 with other cytochrome P450 monooxygenases genes: Pax P (Accession No. AAK11528) of Penicillium paxilli involved in the biosynthesis of paxilline, Itm K (Accession No. AAW88512) of Neotyphodium lolii involved in the biosynthesis of lolitrem, PmP450-2 (Accession No. BAD29968) of Phoma beta involved in the biosynthesis of aphidicolin and P450-4 (Accession No. Q701P2.1) of Gibberella fujikuroi involved in the biosynthesis of gibberellin.

displayed conserved domains of cytochromes P450 monooxygenases (CYP) like the heme-binding loop and the Glu-X-X-Arg motif (Werck-Reichhart and Feyereisen, 2000), and showed no similarities with flavin-containing monooxygenases (FMO).

Alignment of gpe1 with other cytochromes P450 monooxygenases displayed an average identity of 40% to the central and N-terminal parts of enzymes involved in indole diterpene synthesis and in gibberellin synthesis (Figure 3). These enzymes catalyze the addition of hydroxyl groups after cyclization of the diterpenes (Saikia et al., 2008). Replacement of geranylgeranyl diphosphate (diterpene) as a precursor by farnesyl diphosphate (sesquiterpene) can probably lead to the formation of geosmin in a similar process. Farnesyl diphosphate is also an intermediate in geosmin biosynthesis in bacteria (Jiang et al., 2007), and some cyanobacteria have cytochromes P450 monooxygenases involved in the production of sesquiterpenes (Robert et al., 2010). All of these data suggest a possible role of gpe1 protein as a CYP involved in geosmin biosynthesis.

To confirm this hypothesis, the same primers mhsF and mhsR were first used for PCR amplifications in fourteen Penicillium species. The ten geosmin-producing species (including P. expansum) showed the same 1,2 kb PCR product, whereas the four non-producing species gave no signal, or a weaker smaller band (Figure 4).

Therefore the gpe1 gene was functionally characterized in P. expansum M2230, by the gene disruption method. To obtain mutants of gpe1, protoplasts issued from P. expansum M2230 cells were transformed with TopoPhh vector (Figure 2). Forty two transformants which were able to grow on YES medium added with hygromycin were subsequently screened by two consecutive PCRs to...

Figure 5. PCR transformants screening: 1. *P. expansum* wild type with primers *mhsF*/hphR, 2. gpe1 complementary mutant with primers *mhsF*/hphR, 3. Δgpe1 mutant with *mhsF*/hphR, 4. *P. expansum* wild type with primers *hphF*/hphR, 5. gpe1 complementary mutant with primers *hphF*/hphR, 6. Δgpe1 mutant with *hphF*/hphR, M: 1 kb DNA ladder.

monitor the integration of *hph* cassette in the genome of *P. expansum*. Using primer pair *hphF*/hphR, a PCR product of ~0.37 kb (corresponding to *hph* cassette) was obtained in only five transformants (Figure 5, lane 6). These five transformants were then subjected to a second PCR using primers *mhsF* and *hphR*. All gave a ~1.5 kb gpe1/hph fragment (Figure 5, lane 3). No PCR amplification was observed in the wild type *P. expansum*.
with any of the primers combination (Figure 5, lanes 1 and 4).

Geosmin was not detected (limit of quantification 10 ng/L) in each of the mutants, while the production of the wild P. expansum M2230 strain was 14 ng/L.

To produce reverse complements, Δgpe1 mutant protoplasts were transformed with TopoP vector. The transformants which only grew on YES medium but not on YES medium supplemented with hygromycin were selected. These selected transformants were subjected to the same two screening PCRs using primer pairs hphF/hphR and mhsF/hphR. No amplification product in complementary mutants with any of the primer pairs depicts the removal of hph cassette (Figure 5, lanes 2 and 5). Geosmin production by the reverse complements was identical to the production of the wild P. expansum M2230 strain (14 ng/L). So the conclusion of this is the proposition that gene gpe1 encodes a cytochrome P450 monooxygenase involved in the biosynthesis of geosmin.

The fact that the initial DNA fragments p450-1 and p450-2 were isolated from population of transcripts preferentially expressed under patulin-permissive conditions is compatible with our proposition: numerous studies have shown the interactions between different secondary metabolic pathways. If geosmin derives from farnesyl diphasphate, its biosynthesis probably starts with acetyl CoA, via the mevalonate pathway, suggesting concerted regulation process. Such a phenomenon depending on the availability of acetyl CoA was already described for geosmin, with an interaction with doxorubicin synthesis (Singh et al., 2009).

In further studies, the use of gpe1 gene, as a probe, could allow to the characterization of other genes involved in the biosynthetic pathway of geosmin.

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REFERENCES


