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Study of the biosynthesis pathway of the geosmin in *Penicillium expansum*

JURY

M. AZIZ Aziz	Maître de Conférences, Université de Reims Champagne-Ardenne
M. HAFIDI Mohamed	Professeur, Université de Bordeaux
Mme. MATHIEU Florence	Professeur, Université de Toulouse
M. LEBRIHI Ahmed	Professeur, Université de Toulouse

Ecole doctorale : École doctorale: Sciences Ecologiques, Vétérinaires, Agronomiques et Bioingénieries
Unité de recherche : LGC UMR 5503 (CNRS/UPS/INPT)
Directeur de Thèse : Pr. LEBRIHI Ahmed (INP-ENSAT)
Co-Directeur de Thèse : Dr. LIBOZ Thierry (INP-ENSAT)

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Summary

Résumé

La géosmine est un terpénoïde, provoquant un goût moisi-terreux associée à des flaveurs atypiques dans l'eau et le vin. Chez les bactéries, la voie de biosynthèse de la géosmine est bien caractérisée, mais peu de connaissance sont disponibles au sujet de sa biosynthèse chez les eucaryotes, en particulier dans les champignons filamenteux. L'origine de la géosmine dans la vigne est en grande partie attribuable à la présence de *Penicillium expansum* sur les raisins. Dans cette thèse, afin de mieux comprendre la voie de biosynthèse de la géosmine chez *Penicillium expansum*, nous avons décrit la caractérisation et l'analyse de "*gpe1*", un gène codant pour une cytochrome P450 monooxygénase impliquée dans la biosynthèse de la géosmine.

Nous avons démontré que les deux fragments d'ADN: *p450-1* et *p450-2* appartiennent à un seul gène du cytochrome p450 (*gpe1*). La séquence d'acides aminés déduite de *gpe1* a une identité moyenne de 40 % avec les enzymes PbP450-2 et P450-4 qui ont été trouvées impliquées respectivement dans la synthèse d'indole diterpène et dans la synthèse des gibbérellines. Les amplifications par PCR effectuée sur quatorze espèces de *Penicillium* ont montré que seules les espèces producteurices de la géosmine ont donné le même fragment de ~1,2 kb que *gpe1*. L'analyse du gène *gpe1* nous a permis d'identifier la présence de certains domaines conservés de cytochromes P450 monooxygénases. Ensuite, la caractérisation fonctionnelle du gène *gpe1* chez *P. expansum* M2230 a été décrite. Nous avons montré que les mutants de *gpe1* ont perdus leur pouvoir de produire la géosmine alors que les révertants de *gpe1* ont rétablis leur pouvoir de production. Enfin, nous avons démontré qu'une polykétide synthase putative et une putative NRPS sont présentes sur le côté droit du gène *gpe1* proposant que le gène *gpe1* pourrait être une partie d'un "Cluster" codant pour la biosynthèse de métabolites secondaires.

Mots clés: Cytochrome P450 monooxygénase, géosmine, *gpe1*, *Penicillium expansum*.

Abstract

Geosmin is a terpenoid, an earthy-musty compound associated with off-flavors in water and wine. In bacteria, the biosynthesis pathway of geosmin is well characterized, but little is known about its biosynthesis in eukaryotes, especially in filamentous fungi. The origin of geosmin in grapevine is largely attributable to the presence of *Penicillium expansum* on grapes. In this thesis, we have described the characterization and analysis of ~~"~~*gpe1*", a gene encoding a cytochrome P450 monooxygenase probably involved in the biosynthesis of geosmin in *P. expansum* M2230, in order to better understand of the biosynthesis pathway of geosmin in this species.

. We demonstrated that the two DNA fragments i.e. *p450-1* and *p450-2* belong to a single cytochrome p450 gene (*gpe1*). We showed that the deduced amino acid sequence of *gpe1* has an average identity of 40 % with PbP450-2 and P450-4 enzymes which have been found involved in indole diterpene synthesis and in gibberellin synthesis respectively. Then, the results of PCRs performed on the fourteen *Penicillium* species showed that only *Penicillium* species which were producers of geosmin gave the same fragment of ~1.2 kb like *gpe1*. Analysis of the *gpe1* gene enabled us to identify the presence of some conserved domains of cytochromes P450 monoxygenases in the amino acid sequence of *gpe1*. Then, the functional characterization of the *gpe1* gene in *P. expansum* M2230 was described. We illustrated that the mutants of *gpe1* lost their potential to produce geosmin whereas the reverse complements of *gpe1* restored their potential to produce geosmin. Finally, we demonstrated that a putative polyketide synthase and a putative NRPS-like enzyme are present on the right side of the *gpe1* gene suggesting that *gpe1* gene might be the part of a gene cluster encoding the biosynthesis of secondary metabolites.

Key words: Cytochrome P450 monooxygenase, geosmin, *gpe1*, *Penicillium expansum*.

List of Abbreviations

2-MIB:	2-methylisoborneol
aa:	Amino acid
BLAST:	Basic local alignment search tool
bp:	Base pair
CoA:	Coenzyme-A
CPR:	Cytochrome P450 reductase
CTAB:	Cetyltrimethylammonium bromide
CYA	Czapek yeast agar
CYP	Cytochrome P450
DNA:	Deoxyribonucleic acid
EDTA:	Ethylenediaminetetraacetic acid
ER:	Endoplasmatic reticulum
FAD:	Flavine adenine dinucleotide
FCPD:	Fungal cytochrome P450 database
FMN:	Flavin mononucleotide
FPP:	Farnesyl diphosphate
GAC:	Granular activated carbon
GC–MS:	Gas chromatography-mass spectrometry
GGPP:	Geranylgeranyl diphosphate
hph:	Hygromycin B phosphotransferase
LQ:	Limit of quantification
MEA:	Malt Agar Extract
NADPH:	Nicotinamide adenine dinucleotide phosphate (reduced form)
NRPS:	Non-ribosomal peptide synthase
OD:	Optical Density
PAC:	Powdered activated carbon
PCR:	Polymerase chain reaction
PKS:	Polyketide synthase
RNA:	Ribonucleic acid
rpm:	Rotation per minute

SDS: Sodium dodecyl sulfate

TOC: Total organic carbon

UV: Ultraviolet

YES: Yeast extract sucrose

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Chapter I

Literature Review

1. Literature Review

1.1. Fungal Secondary Metabolite

The primary metabolism of an organism is the summation of an interrelated series of enzyme-catalyzed chemical reactions (both degradative and synthetic) which provide the organism with its energy, its synthetic intermediates and its key macromolecules such as protein and DNA. On the other hand, secondary metabolism involves mainly synthetic processes whose end-products, the secondary metabolites, play no obvious role in the internal economy of the organism.

Plants and microorganisms produce a vast number of natural compounds known as secondary metabolites. Kossel (1891) introduced the concept of ―secondary metabolites" to distinguish these compounds from primary metabolites that these products are not necessary for the growth, survival or reproduction of their producers. Secondary metabolites are substances of limited molecular weight (normally < 3000 Daltons) which display an enormous structural diversity. However each of them is synthesized only by a limited taxonomic group of organisms whereas primary metabolites are found in all living organisms since they perform essential functions in growth and development.

Many species in the fungal kingdom have unique and unusual biochemical pathways. Important pharmaceuticals such as penicillin, cyclosporin and statins; potent poisons, including aflatoxins and trichothecenes; and some Janus-faced metabolites that are both toxic and pharmaceutically useful, such as the ergot alkaloids are the products of these pathways. All of these natural products, along with many other low-molecular-weight fungal metabolites, are classified together as secondary metabolites. Secondary metabolites are produced as families of related compounds at restricted parts of the life cycle, with production often correlated with a specific stage of morphological differentiation. Secondary metabolites have restricted taxonomic distribution as only a small group of organisms

produces each metabolite and the producer organisms can grow without synthesizing these metabolites. Secondary metabolites are, often synthesized after active growth has ceased, which do not have an obvious function in producer species (Keller *et al.*, 2005).

In fungi, in the case of the Basidiomycetes and the larger Ascomycetes, secondary metabolites may be obtained simply by extraction of the organism collected in the field. But the great advantage of the fungi as sources of secondary metabolites is their ability to produce the compounds on aqueous media. As a result, secondary metabolites of diverse type are conveniently available in the laboratory for chemical, biochemical and biological studies, and a few are manufactured on a commercial scale. In some cases the same secondary metabolites have been obtained from fruiting-bodies and from aqueous culture of Basidomycetes, though in most cases the compounds have so far only been obtained from one of the sources. The laboratory cultures of Basidiomycetes are, of course, mycelial; Basidiomycetes do not normally form fruiting-bodies under laboratory conditions and in some cases have resisted all attempts to induce them to do so. In aqueous cultures, secondary metabolites accumulate both in the medium and in the mycelium. For related compounds, the distribution between medium and mycelium can often be correlated with water-solubility, though this apparent correlation may be a result of some other factor such as ease of transport across cell membranes (Turner, 1971).

Fungal secondary metabolites encompass over 30,000 known compounds with an extremely diverse array of chemical structures. It is intriguing that all these secondary metabolites originate from a few common biosynthetic pathways utilizing precursors (small biosynthetic units or building blocks) formed during primary metabolism. The intermediates resulting from condensation of these small biosynthetic units are further elaborated (~~–t~~ailored" or ~~–d~~ecorated") by numerous enzyme-catalyzed reactions, leading to products with a diversity of structures. Thus, fungal secondary metabolites are conveniently classified

based on their biosynthetic origin as polyketides (e.g. aflatoxin and fumonisins), nonribosomal peptides (e.g. sirodesmin, peramine and siderophores such as ferricrocin), terpenes (e.g. T-2 toxin, deoxynivalenol (DON)), and indole alkaloids (e.g. paxilline, fumigaclavines and fumitremorgens) (Keller *et al.*, 2005; Gunatilaka, 2006).

Biosynthesis of fungal secondary metabolites often involves elaborate biochemical pathways and is regulated by a group of genes known as biosynthetic genes. The insights that have been gained from recent advances in genetics, genomics, molecular biology, and bioinformatics have contributed to the understanding and manipulation of these genes for improved production, or inhibition of production, of fungal secondary metabolites.

Fungal secondary metabolites are well known for their biological activity and represent some of today's important and useful pharmaceuticals and agrochemicals. Among the pharmaceuticals, most noteworthy are penicillins, cephalosporins, and fusidic acid with antibacterial activity; echinocandin B, pneumocandins, griseofulvin, and strobilurins with antifungal activity; integric acid and integresone with antiviral activity; cyclosporin A and mycophenolic acid with immunosuppressive activity; fumagillin and rhizoxin with antitumor activity; lovastatin and pravastatin with cholesterol-lowering activity; and ergot alkaloids (for example, ergotamine) with antimigraine activity. Gibberellins and zearalenones are fungal secondary metabolites used in agriculture as plant growth hormones and in animal husbandry as growth promoters, respectively. Some fungal secondary metabolites such as mycotoxic aflatoxins and mutagenic fusarin C possess potent toxic and carcinogenic activities and are therefore important in human, animal, and plant health (Vining, 1990; Fox and Howlett, 2008) whereas some volatile non-toxic secondary metabolites such as geosmin and 2-methylisoborneol (2-MIB) haves also concerns for humans as they are responsible for off-flavors in drinking water and wines (Gerber, 1979; Darriet *et al.*, 2000).

In some fungi, secondary metabolism (the process that results in the production of secondary metabolites) has been found to commence during the stationary or resting phase of their development and is often associated with sporulation and colony formation. Some well-documented functions of fungal secondary metabolites include enhancement of spore survival by acting as virulence factors and protecting against ultraviolet (UV) light, and augmentation of their fitness and competitive ability against other fast-growing organisms. Fungal metabolites associated with sporulation may activate sporulation (for example, linoleic acid analogs produced by *Aspergillus nidulans*), provide pigmentation required for sporulation structures (for example, melanins produced by *Alternaria alternata*), or have toxic properties to ward off competing organisms (for example, mycotoxins produced by some *Aspergillus* species). The relationship between production of secondary metabolites and regulation of asexual sporulation by a G-protein–mediated growth pathway in *Aspergillus* species was established over a decade ago. Also, it has been speculated that secondary metabolites in fungi function as metal chelators (combining with metal ions and removing them from their sphere of action), which is important in mineral nutrition, and that pathways leading to their formation act as safety-valve shunts that prevent the accumulation of toxic intermediates of primary metabolism under conditions of unbalanced growth (Calvo *et al.*, 2002; Fox and Howlett, 2008).

1.2. *Penicillium expansum*

1.2.1. Classification and morphological description

Penicillium expansum is the typical fungus of the genus *Penicillium* and is therefore also one of the most studied species in the genus (Pitt, 1979). This fungus belongs to phylum Ascomycota, class Eurotiomycetes, subclass Eurotiomycetidae, order Eurotiales and family Trichocomaceae.

After 7 days of incubation on Petri dish containing CYA (Czapek Yeast Agar) medium at 25 °C, a colony of *P. expansum* attains a diameter of 26-50 mm, on MEA (Malt Agar Extract) medium, its colony could be 16-34 mm in diameter, while on YES (Yeast Extract Sucrose) agar medium, the diameter of a colony could be 38-65 mm (Figure 1) but there is no growth at 37 °C (Frisvad and Samson, 2004). Cultural characteristics of this fungus include: the colonies grow rapidly on the culture media, with radial wrinkles up to 2 mm deep, spore heavily, very variable, from velvety with conidiophores occurring singly to granular with conidiophores grouped together in fascicles or producing quite distinct coremia, often showing radial zonation; white, rapidly becoming dull yellow green to greyish green with the production of conidia (Figure 2); reverse variable, colorless to yellow brown to deep brown. The conidial heads are asymmetric, once or twice branched, elongate, bearing long tangled chains of conidia. The conidiophores are smooth or in some strains slightly roughened, moderately long, up to 400 µm long but occasionally up to 600-700 µm long and 3-3.5 µm wide; branches 15-25 x 2.5-3.5 µm, occasionally longer. But the metulae arising from branches at about the same level, 3 to 6 in number, and about 10-15 x 2-3 µm. The phialides are in groups of 5-9, often about 8-12 x 2-2.5 µm, occasionally longer. The conidia are smooth, elliptical to cylindrical when first formed and usually remain elliptical, generally 4-5 x 2.5-3.5 µm (Link, 1809; Onions, 1966) (http://www.mycobank.org/MycoTaxo.aspx?Link=T&Rec=159382).

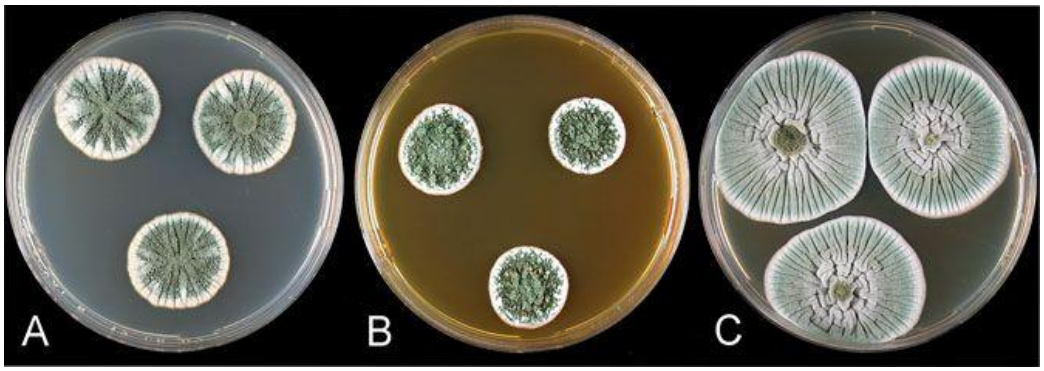


Figure1: *Penicillium expansum*, A-C. 7-days-old colonies on A. CYA, B. MEA, C. YES at 25 °C.

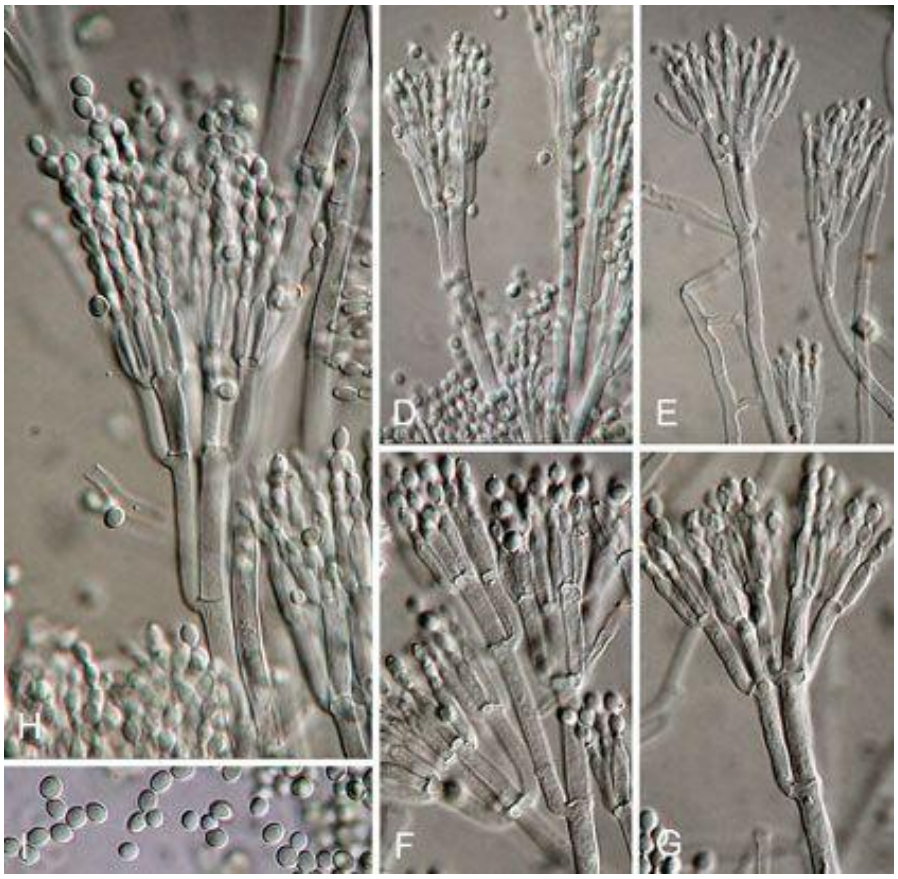


Figure 2: *Penicillium expansum*, D-H. Conidiophores. I. Conidia. Scale bar = 10 μm.

1.2.2. Host

Penicillium expansum is commonly present in soil and in a wide variety of organic material including grains and cereal products, and though generally isolated from mouldy fruit, particularly apples, it also occurs on other pomaceous fruits such as cherries, peaches, pears, grapes, olives, pineapple and sometimes on citrus and avocado. It is also common on walnuts, pecans, hazelnuts and acorns. *P. expansum* is responsible for the postharvest decay of these fruits leading to important economic losses in the fruit industry (Filtenborg *et al.*, 1996; Karabulat *et al.*, 2002; Karabulat and Bakyal, 2002; Venturini *et al.*, 2002).

1.2.3. Secondary metabolites produced by *P. expansum*

P. expansum has been reported to produce many secondary metabolites such as: chaetoglobosins A and C, communesin B which are cytotoxic metabolites (Bridge *et al.*, 1989; Frisvad and Filtenborg, 1989; Frisvad, 1992; Andersen *et al.*, 2004), the bioactive compounds expansolides A and B (Massias *et al.*, 1990; Andersen *et al.*, 2004), an antibiotic penicillic acid (Leistner and Pitt, 1977), roquefortine C which is neurotoxic (Frisvad and Filtenborg, 1983; Bridge *et al.*, 1989), patulin which is carcenogenic, citrinin which is nephrotoxic (Leistner and Pitt, 1977; Frisvad and Filtenborg, 1983; Paterson *et al.*, 1987; Andersen *et al.*, 2004) and geosmin which is an aromatic volatile secondary metabolite (Mattheis and Roberts, 1992). Among the above mentioned extrolites of *P. expansum*, chaetoglobosins A and C, penicillic acid, patulin and citrinin belong to polyketides. Geosmin and expansolides A and B are terpenes whereas roquefortine C and communesin B belong to indole alkaloid family.

In literature, it has been reported that citrinin biosynthesis was originated from a pentaketide in *Penicillium* and *Aspegillus* species (Barber and Staunton, 1980; Sankawa *et al.*, 1983). It was demonstrated in literature that in the genus *Aspergillus*, the condensation

of one acetyl coenzyme A (acetyl-CoA) molecule with four malonyl-CoA molecules, followed by the addition of three methyl units has synthesized the citrinin (Colombo *et al.*, 1981; Hill *et al.*, 1981). In the contrary, Hajjaj *et al.* (1999) revealed that citrinin is formed from a tetraketide precursor arising from the condensation of one acetyl-CoA molecule with three malonyl-CoA molecules in the filamentous fungus *Monascus ruber* instead of a pentaketide as reported in *Penicillium* and *Aspergillus.* The patulin production pathway from the polyketide, 6-methylsalicylic acid (6-MSA) has been established and is thought to involve at least 10 different enzymatic steps (Moake *et al.*, 2005). However, two of the genes namely the 6-methylsalicylic acid synthase (6-msas) gene (Beck *et al.*, 1990) and the isoepoxydon dehydrogenase gene (idh) (Gaucher & Fedeshko, 2000) encoding these enzymes have been cloned and sequenced, both from *Penicillium urticae*. Precursor feeding experiments revealed that tryptophan, histidine, and mevalonate are involved in the biosynthesis of roquefortine C (Barrow *et al.*, 1979; Gorst-Allman *et al.*, 1982). Garcia-Estrada *et al.* (2011) cloned 5 genes from a single gene cluster of *Penicillium chrysogenum* involved in the biosynthesis and secretion of the mycotoxin roquefortine C and proved that the roquefortine C derive from a single pathway. Communesins are of mixed biosynthetic origin, predictably derived from tryptophan, mevalonate, acetate and a methyl group from methionine (Wigley *et al.*, 2006). In bacteria, MEP or/and MVA pathway may lead to the synthesis of the geosmin (Dickschat *et al.*, 2005; Jüttner and Watson, 2007). Biosynthesis of the geosmin has been discussed in detail in the next part.

The cytochtochromes P450 monooxygenases could be involved in the biosynthesis of geosmin in *P. expansum*. Therefore, an inclusive introduction of these enzymes has been given in the following section.

1.3. Cytochrome P450 monooxygenase

1.3.1. Charecteristics of cytochrome P450s

Cytochrome P450 (CYP) genes encode a superfamily of heme-thiolate-containing enzymes. These enzymes are found in all life forms from prokaryotes (archea, bacteria) and lower eukaryotes (fungi and insects) to higher eukaryotes (plants and animals including humans) (Cresnar and Petric, 2011) and reported to be involved in an array of diverse endogenous and exogenous oxidative processes (Guengerich, 1991). Cytochromes P450 are external monooxygenases. Monooxygenases (mixed function oxidases) catalyse the incorporation of a single atom of molecular oxygen into a substrate with the concomitant reduction of the other atom to water. There are two classes of monooxygenases: the internal and the external monooxygenases. Their character as hemoproteins and their unusual spectral properties displaying a typical absorption maximum of the reduced CO-bound complex at 450 nm gave them a name as cytochromes P450: cytochrome stands for a hemoprotein, P for pigment and 450 reflects the absorption peak of the CO complex at 450 nm. The following reaction is catalysed by cytochrome P450 systems:

$$RH + O_2 + NAD(P)H + H+ \rightarrow ROH + H_2O + NAD(P)+$$

A separate electron donating system donates the electrons needed for the oxygen insertion in the substrate molecule (R). The electron donating system is either a two-protein system (adrenodoxin and adrenodoxin reductase) for mitochondrial and prokaryotic P450s or a single protein (cytochrome P450 reductase, CPR) for cytochrome P450 enzymes that are located in the endoplasmatic reticulum (ER). Most fungal cytochrome P450s identified thus far are expected to be located in the ER.

1.3.2. Structure of P450

Three dimensional structures of cytochrome P450s have shown somewhat similarity although cytochrome P450 amino acid sequences are not well conserved between different

families. The conserved structures of cytochrome P450s include the heme binding region at the C-terminus of the protein and the putative substrate binding region (Figure 3) (van den Brink *et al.*, 1998). An additional N-terminal peptide is present in eukaryotic endoplasmic reticulum (ER) localized cytochrome P450s. This noncleavable signal peptide is responsible for the localization in the ER membrane.

The other component of the cytochrome P450 enzyme system is cytochrome P450 reductase (CPR). This protein is able to reduce cytochrome P450 enzymes. CPR is a flavoprotein of about 78 kDa, containing 1 mol each of the prosthetic factors FAD (flavine adenine dinucleotide) and FMN (flavin mononucleotide) per mole protein (Figure 3). CPR consists of a small membrane spanning region of 6 kDa (TR) and a hydrophilic, cytoplasmatic part of approximately 72 kDa (Black *et al.*, 1979). The hydrophilic part can be divided into four structural domains interacting with the cytochrome P450, NADPH, and the cofactors FAD and FMN (Porter and Kasper, 1986; Shen *et al.*, 1989). The cofactors are important for the electron flow from NADPH to FAD to FMN and finally to the electron acceptor cytochrome P450 (Vermillion *et al.*, 1981; Kurzban and Strobel, 1986; Porter, 1991).

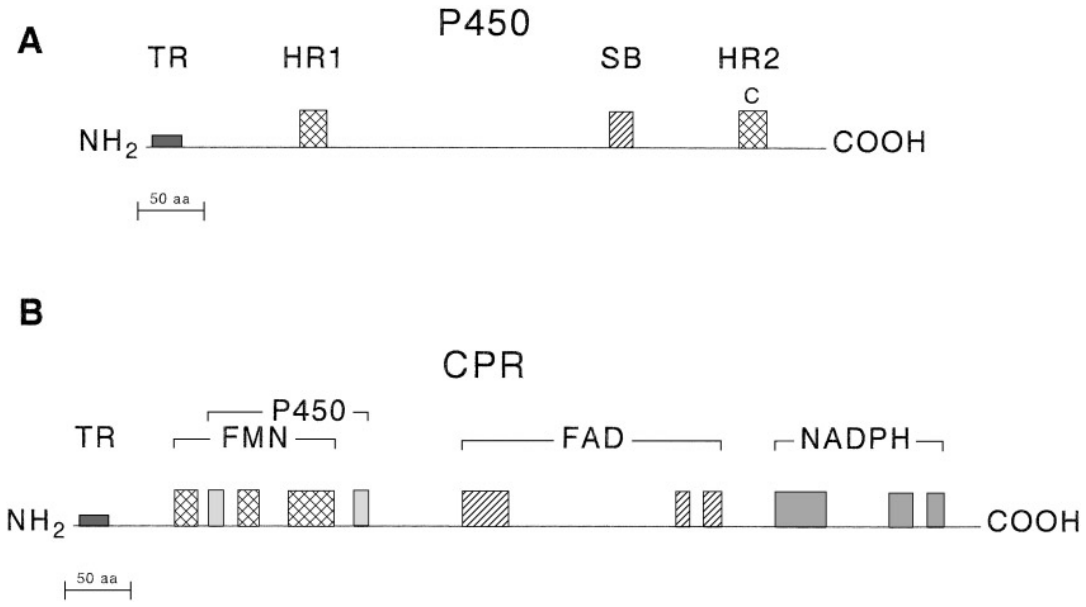


Figure 3. Schematic representation of the eukaryotic endoplasmatic reticulum type cytochrome P450 enzyme system (van den Brink *et al.*, 1998). (A) Cytochrome P450. Indicated are the membrane-spanning domain (TR), the two regions involved in heme binding (HR1 and HR2), and the completely conserved cysteine residue (C). SB indicates the putative substrate binding region. (B) Cytochrome P450 reductase (CPR). The transmembrane region is indicated by TR. FMN and FAD indicate regions involved in the interaction with these prosthetic factors. P450 indicates charged regions putatively involved in interaction with cytochrome P450 enzymes and NADPH indicates the region involved in NADPH binding and recognition.

1.3.3. Reactions catalyzed by P450s

They are found involved in reactions as diverse as e.g. hydroxylation, N-, O- and S-dealkylation, sulphoxidation, epoxidation, deamination, desulphuration, dehalogenation, peroxidation, and N-oxide reduction. More than 20 different reactions, which can be catalysed by cytochromes P450s have been listed: hydrocarbon hydroxylation, alkene epoxidation, alkyne oxygenation, arene epoxidation, aromatic hydroxylation, N-dealkylation, S-dealkylation, O-dealkylation, N-hydroxylation, N-oxidation, S-oxidation, oxidative deamination, oxidative dehalogenation, alcohol and aldehyde oxidations, dehydrogenation, dehydrations, reductive dehalogenation, N-oxide reduction, epoxide reduction, reductive β-scission of alkyl peroxide, NO reduction, isomerizations, oxidative C-C bond cleavage (Sono *et al.*, 1996). They have different substrates as: fatty acids, steroids, prostaglandins, as well as a multitude of foreign compounds such as drugs, anaesthetics, organic solvents, ethanol, alkylaryl hydrocarbon products, pesticides, and carcinogens.

1.3.4. Involvement of P450s in biosynthesis of secondary metabolites and different functions

33 cytochromes P450 (CYPs) and 18 CYP genes have been identified in *Streptomyces avermitilis* and *Streptomyces coelicolor* A3, respectively. At least one-third of them were proposed to be involved in the biosynthesis of secondary metabolites, in both organisms. The probable contribution of many of the remaining CYP genes to secondary metabolite production was also reported but they were not linked to a specified gene cluster (Lamb *et al.*, 2003). In literature, cytochrome P450 enzymes have been reported to involve in many metabolic pathways, including terpenes and their derivatives (Nelson *et al.*, 1993; Werck-Reichhart and Feyereisen, 2000; Bernhardt, 2006). White *et al.* (2006) have cloned and characterized part of two putative cytochrome P450 monooxygenase genes *P-450 1* and *P-450 2* in *Penicillium expansum*. They reported the involvement of these genes in patulin

biosynthesis as their increased expression was observed under patulin-permissive conditions. Saikia *et al.* (2007) demonstrated the involvement of two cytochrome P450 monooxygenases, PaxP and PaxQ in paxilline biosynthesis in *Penicillium paxilli*. Cytochrome P450 enzymes have been reported involved in many complex fungal bioconversion processes (van den Brink *et al.*, 1998). The conversion of hydrophobic intermediates of primary and secondary metabolic pathways of fungi is catalyzd by cytochrome P450 monooxygenases. They also detoxify natural and environmental pollutants and allow fungi to grow under different conditions. 4,538 putative P450 genes have been identified in the genomes of 66 fungal and 4 oomycete species. The systematic identification and multifaceted analyses of P450s at multiple taxon levels via the web are facilitated by the Fungal Cytochrome P450 Database (FCPD). All data and functions are available at the web site http://p450.riceblast.snu.ac.kr/ (Park *et al.*, 2008).

1.4. Geosmin

1.4.1. General characteristics

Geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol) (Figure 4) is a small aromatic volatile secondary metabolite responsible for the characteristic odor of freshly plowed earth and belongs to the class of sesquiterpenes. The name geosmin is derived from two Greek words: ―ge" meaning earth and ―osme" meaning odor. This compound was first isolated by Gerber and Lechevalier in 1965 (Gerber and Lechevalier, 1965). Geosmin exists as (+) and (–) enantiomers and odor outbreaks are caused by biological production of the naturally occurring (–) enantiomers which are some 10 times more potent than the (+) molecules (Watson *et al.*, 2007). The molecular formula of geosmin is $C_{12}H_{22}O$ having a molecular mass of 182.3 g / mol. Geosmin is responsible for undesirable musty or off-flavors in drinking water, wine, grape juices, fish and other food stuffs (Gerber, 1979; Heil and Lindsay, 1988; Darriet *et al.*, 2000; La Guerche *et al.*, 2005). Geosmin has been identified,

often associated with another volatile secondary metabolite i.e 2-methylisoborneol (2-MIB) which is also found responsible for the earthy/musty smell (Buttery and Garibaldi, 1976).

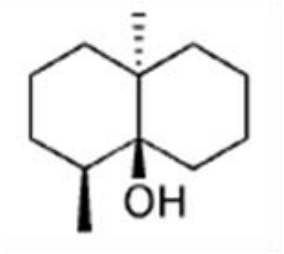


Figure 4. Chemical structure of geosmin

1.4.2. Production of geosmin by microorganisms

Geosmin can be produced by a wide variety of microorganisms (Table 1). The actinomycetes: *Streptomyces coelicolor*, *S. avermitilis, S. peucetius* and *S. griseus*, which are a complex group of bacteria present in a wide variety of environments produce geosmin (Gerber, 1971; Zaitlin and Watson 2006). Several species of cyanobacteria e.g. *Oscillatoria simplicissima* and *Anabaena scheremetievi* were found producing geosmin (Izaguirre *et al.*, 1982). Dickschat *et al.* (2004) found that the characteristic odor of the myxobacterium *Myxococcus xanthus* was due to the geosmin. Geosmin is notably found in drinking water (Gerber, 1979) and grape juice (Darriet *et al.*, 2000; 2001). In case of water, contamination is strictly bacterial as geosmin is produced by several groups of benthic and pelagic aquatic microorganisms, mainly cyanobacteria and actinomycetes which are found in eutrophic surface waters such as drinking water reservoirs (Jüttner and Watson, 2007). Two groups of superior fungi have been reported to produce geosmin: some basidiomycetes species (*Cortinarius herculeus*, *Cystoderma amianthinum*, and *Cy. carcharias*) (Breheret *et al.*, 1999) and various species of *Penicillium*, such as *P. citrinum* (Pisarnitskii and Egorov, 1988), *P. expansum* (Mattheis and Roberts, 1992), *P. vulpinum* (Börjesson *et al.*, 1993), *P.*

aethiopicum, *P. clavigerum*, *P. discolor*, *P. echinulatum*, *P. formosanum*, *P. hirsutum*, and *P. roqueforti* (Larsen and Frisvad, 1995). Spiteller *et al.* (2002) demonstrated the synthesis of geosmin by the liverwort *Fossombronia pusilla*. Lu *et al.* (2003) concluded that the red beets (*Beta vulgaris* L.) are capable of endogenous synthesis of geosmin. Hayes *et al.* (1991) found the amoeba *Vannella* as potential producers of the geosmin. Geosmin can also be synthesised by insects. Omura *et al.* (2002) explained that the small millipede *Niponia nodulosa* (Polydesmida: Cryptodesmidae) emits geosmin when disturbed.

In a study forty-three *Penicillium*-related species isolated from rotten grapes of the Bordeaux vineyards have been analyzed by gas chromatography-mass spectrometry (GC–MS) for their geosmin production. It was found that all strains producing geosmin belonged to only one species i.e. *Penicillium expansum* (La Guerche *et al.*, 2004). Its presence in juice obtained from rotten grapes suggested that *Penicillium expansum* that developed on the grapes contributed to the presence of geosmin in wines. La Guerche *et al.* (2005) demonstrated the necessary and complementary action of *Botrytis cinerea* and *Penicillium expansum* in geosmin production in grape juice and in crushed grape berries. *Botrytis cinerea* was largely present in earthy grapes. The authors illustrated that *P. expansum* alone was able to produce geosmin on a model medium but not on grapes, but the grape juice became favourable to geosmin production by *P. expansum* after 7 days pre-culture of some *B. cinerea* strains on this juice. La Guerche *et al.* (2007) reported that two groups of strains of *B. cinerea* ([bot +] and [bot ⁻]) induced significantly higher production of geosmin from *P. expansum*. Morales-Valle *et al.* (2011) also demonstrated that some of *B. cinerea* strains induced detectable geosmin production on grape broth medium by *P. expansum*. So, in the case of wine, origin of geosmin is mainly due to the development of *Penicillium expansum* and/or a combination of *P. expansum* and *B. cinerea* strains on grapes.

Table 1: Production of geosmin by different organisms.

Organisms	Species	References
Bacteria	*Streptomyces oelicolor*	Jiang *et al.*, 2006
	Streptomyces vemitilis	Cane *et al.*, 2006
	Streptomyces peucetius	Ghimire *et al.*, 2008
	Streptomyces griseus	Whitmore and Denny, 1992
Cyanobacteria	*Oscillatoria plicissima*	Izaguirre *et al.*, 1982
	Anabaena heremetievi	
Myxobacteria	*Myxococcus xanthus*	Dickschat *et al.*, 2004
Fungi	*Penicillium expansum*	Mattheis and Roberts, 1992
		La Guerche *et al.*, 2004
	P. citrinum	Pisarnitskii and Egorov, 1988
	P. vulpinum	Börjesson *et al.*, 1993
	P. aethipicum	Larsen and Frisvad, 1995
	P. clavigerum	
	P. sclerotiorum	
	P. discolor	
	P. echinulatum	
	P. formosanum	
	P. hirsutum	
	P. roqueforti	
	Cortinarius herculeus	Breheret *et al.*, 1999
	Cystoderma ianthinum	
	Cystoderma archarias	
Liverwort	*Fossombronia pusilla*	Spiteller *et al.*, 2002
Red beets	*Beta vulgaris L.*	Lu *et al.*, 2003
Millipede	*Niponia nodulosa*	Omura *et al.*, 2002

1.4.3. Different methods to analyze geosmin

In general, the human taste and odor sensitivity threshold for geosmin is extraordinary low as 10 ng / L (Cook *et al.*, 1991; Suffet *et al.*, 1999; Watson *et al.*, 2000). To date, numerous methods to analyze geosmin are available. Johnsen and Kuan (1987) described a simple and rapid method for the extraction and quantification of geosmin from pond water and microbial culture media. They used methylene chloride extraction and gas chromatography (GC) eliminating the costly stripping devices. This procedure has approximately 65 % recovery efficiency. Darriet *et al.* (2000) performed quantification of geosmin by gas chromatography–mass spectrometry (GC/MS) using the HP5890-I-HP5970 mass selective detector, in the selected ion monitoring mode (SIM). Benanou *et al.* (2003) analyzed geosmin in water samples by stir bar sorptive extraction (SBSE) followed by on-line thermal desorption (TD) capillary gas chromatography–mass spectrometry (GC/MS). Quantification was performed using the MS in the single-ion-monitoring mode (SIM) with 2,4,6-trichloroanisole-D5 as internal standard. Quantification limit was 0.5 ng / L and more than twenty samples per day can be analyzed by this technique. A method constituting micro extraction and gas chromatography-mass spectrometry with limit of quantification as 15.6 ng / L was used for the analysis of geosmin in grape juice samples inoculated with *P. expanusm* and *B. cinerea* (Moralles-Valle *et al.*, 2010).

For rapid, selective, and sensitive analysis of geosmin and 2-methyisoborneol, a pre-concentration step is usually necessary. Among the extraction / enrichment techniques (Table 2), closed loop-stripping analysis (CLSA) and some of its modified versions have been the most frequently used method for geosmin and 2- MIB analysis (Zander and Pingert, 1997; Hassett and Rohwer, 1999). Also, liquid–liquid extraction (LLE) (Wood and Snoeyink, 1977; Shin and Ahn, 2004; Xiaoyan *et al.*, 2007), solid phase extraction (SPE) (Xiaoyan *et al.*, 2007), solid phase microextraction (SPME) (Lloyd *et al.*, 1998; Watson *et*

al., 2000; Nakamura and Daishima, 2005; Sung *et al.*, 2005; Boutou and Chatonnet, 2007), purge and trap (PT) (Salemi *et al.*, 2006), stir bar sorptive extraction (SBSE) (Nakamura *et al.*, 2001), and recently headspace single drop microextraction (SDME) (Bagheri and Salemi, 2006) have been developed. All of the above mentioned techniques present some drawbacks. CLSA, PT, SPME and SBSE use expensive materials, are time-consuming and usually have carryover effects. Furthermore, SPME and SBSE have long-time sorbent conditioning. On the other hand, LLE and SPE use large amounts of potentially toxic and normally expensive organic solvents, time-consuming and the high manipulation of the sample can lead to undesirable contaminations. In the case of SDME fast stirring speed and air bubbles cause a drop instability and tend to break up the organic drop, and equilibrium could not be attained after a long time in most cases.

Table 2: Extraction / enrichment techniques used to preconcentrate geosmin prior to quantification by gas chromatography-mass spectrometry.

Methods	References
Closed loop-stripping analysis (CLSA)	Zander and Pingert, 1997; Hassett and Rohwer, 1999
Liquid–liquid extraction (LLE)	Wood and Snoeyink, 1977; Shin and Ahn, 2004; Xiaoyan *et al.*, 2007
Solid phase extraction (SPE)	Xiaoyan *et al.*, 2007
Solid phase microextraction (SPME)	Nakamura and Daishima, 2005; Boutou and Chatonnet, 2007
Stir bar sorptive extraction (SBSE)	Nakamura *et al.*, 2001
Headspace single drop microextraction (SDME)	Bagheri and Salemi, 2006
Ultrasound-assisted dispersive liquid–liquid microextraction (USADLLME)	Cortada *et al.*, 2011

Cortada *et al.* (2011), therefore, to overcome the above mentioned drawbacks has developped a fast, simple and environment friendly ultrasound-assisted dispersive liquid–liquid microextraction (USADLLME) procedure to preconcentrate geosmin and 2-methylisoborneol from water and wine samples prior to quantification by gas chromatography-mass spectrometry. The use of ultrasound energy to disrupt the extractant phase reduces the consumption of organic solvent because the disperser solvent is not needed, being ultrasound-assisted dispersive liquid–liquid microextraction (USADLLME) a more environment friendly technique.

1.4.4. Treatments to control geosmin

Different physical, chemical and biological treatments exist to control the earthy-musty odors in public water supplies (Table 3). Geosmin is relatively stable to chemical (Westerhoff *et al.*, 2006; Peter and Von Gunten, 2007) and biological degradation and can persist in the open water in the dissolved form for some time. This is an important factor to consider when attempting to understand and trace the distribution, transport, and fate of this volatile organic compound in aquatic systems and its response to water treatment. Dissolved geosmin is slowly degraded by bacterioplankton in oxic freshwater (Durrer *et al.*, 1999), but little is known about the fate of this compound under anoxic water conditions.

Kutschera *et al.* (2009) investigated the degradation of geosmin by UV irradiation at different wavelengths under varying boundary conditions. They found that conventional UV radiation (254 nm) is ineffective in removing this compound from water. In contrast to the usual UV radiation, UV/VUV radiation (254 + 185 nm) that creates advanced oxidation conditions was more effective in the removal of the taste and odor compound in ultrapure as well as in raw water. Additionally, they also studied the formation of the byproduct nitrite. In the UV/VUV irradiation process up to 0.6 mg / L nitrite was formed during the complete photo initiated oxidation of the odor compounds. However, the addition of low ozone doses

as 20 µg / min (with ozone concentration 5 µg / L) could prevent the formation of nitrite in the UV/VUV irradiation experiments.

Geosmin can effectively be removed to concentrations below than 10 ng / L by powdered activated carbon (PAC) when the correct dose is applied. Powdered activated carbon is often used in treatment plants for the mitigation of problem odors e.g. produced by geosmin and 2-MIB, as it is relatively inexpensive and can be applied only when required (Cook *et al.*, 2001). Elhadi *et al.* (2004) conducted experiments using two parallel filter columns containing fresh and exhausted granular activated carbon (GAC) / sand media. The GAC media was exhausted in terms of total organic carbon (TOC) removal. Typical ozonation by-products were fed to the filters along with the target odor compounds in order to simulate the effect on a biofilter of upstream ozonation. Ozonation alone can at least partially destroy geosmin with removals being dependent on transferred ozone dose and water characteristics (e.g. availability of OH radical precursors). Biofiltration following ozonation has the potential to further significantly reduce the concentration of these highly degradable ozonation products. Additionally, higher biological compounds formed by ozonated water will increase biomass in the filter and thereby enhance the biofilter's ability to degrade the residual geosmin as well as reducing the biological instability. 76 to 100 % geosmin was removed using fresh granular activated carbon whereas the removal of geosmin was initially less using the exhausted granular activated carbon but removal increased over time. So, the use of biofiltration following ozonation as a means of geosmin removal is quite encouraging.

Hoefel *et al.* (2009) reported the isolation of a Gram-negative bacterium, Geo48, from the biofilm of a water treatment plant (WTP) sand filter and demonstrated to degrade geosmin. Eaton and Sandusky (2010) identified two terpene-degrading bacteria: *Pseudomonas* sp. SBR3-tpnb and *Rhodococcus wratislaviensis* DLC-cam which convert

(+/–)-geosmin to several oxidation products; the major products are ketogeosmins which have no odors.

Presence of geosmin is highly detrimental to the aromatic quality of wines due to its low olfactory perception threshold and stability during aging (Darriet *et al.*, 2000; 2001). Removal or degradation processes will be detrimental to the organoleptic quality of wines, and cannot be applied. Nowadays, predictive models of fungal growth are therefore the best way to control geosmin production. Judet-Correia *et al.* (2010) validated a model for predicting the combined effect of temperature and water activity on the radial growth rate of *Botrytis cinerea* and *Penicillium expansum* on grape berries. This approach allowed validation of the model over a wide range of variation of temperature and water activity, but also the estimation of the optimal growth rate on grape berries under non optimal conditions. This facility was particularly useful for the examination of fast growing fungi on small fruits.

Table 3: Different treatments to control the geosmin in water.

Treatment	Principle	References
UV/VUV irradiation	by photoinitiated oxidation	Kutschera *et al.*, 2009
Powdered activated carbon	by adsorption	Cook *et al.*, 2001
Biofiltration followed by ozonation	increased biomass in the filter by ozonation enhances the biofilter's ability to degrade the geosmin	Elhadi *et al.*, 2004
Terpene-degrading bacteria	by conversion of geosmin to ketogeosmin	Eaton and Sandusky, 2010

1.4.5. Biosynthesis pathway of geosmin

1.4.5.1. Biochemical pathway of geosmin synthesis in bacteria

Farnesyl diphosphate (FPP) is the immediate precursor of cyclic sesquiterpenes (Cane *et al.*, 2006). Geosmin was produced by *Streptomyces* when labeled 1-deoxy-D-xylulose (Spiteller *et al.*, 2002) was added, while labeled mevalolactone and leucine were applied successfully with the myxobacteria *Myxococcus xanthus* and *Stigmatella aurantica* (Dickschat *et al.*, 2005). From these studies, it is evident that there are several different biosynthetic pathways of isoprenoid synthesis in microorganisms, one or more of which may lead to the production of geosmin by different taxa (Figure 5). In many bacterial groups, the MEP pathway is the major biosynthetic isoprenoid route; nevertheless, there is some evidence that the MVA pathway is also used. The MVA pathway may function exclusively in the synthesis of geosmin and other isoprenoids in some groups such as myxobacteria and contribute to geosmin production in the stationary growth phase of streptomycetes (Seto *et al.*, 1998; Dickschat *et al.*, 2005; Jüttner and Watson, 2007).

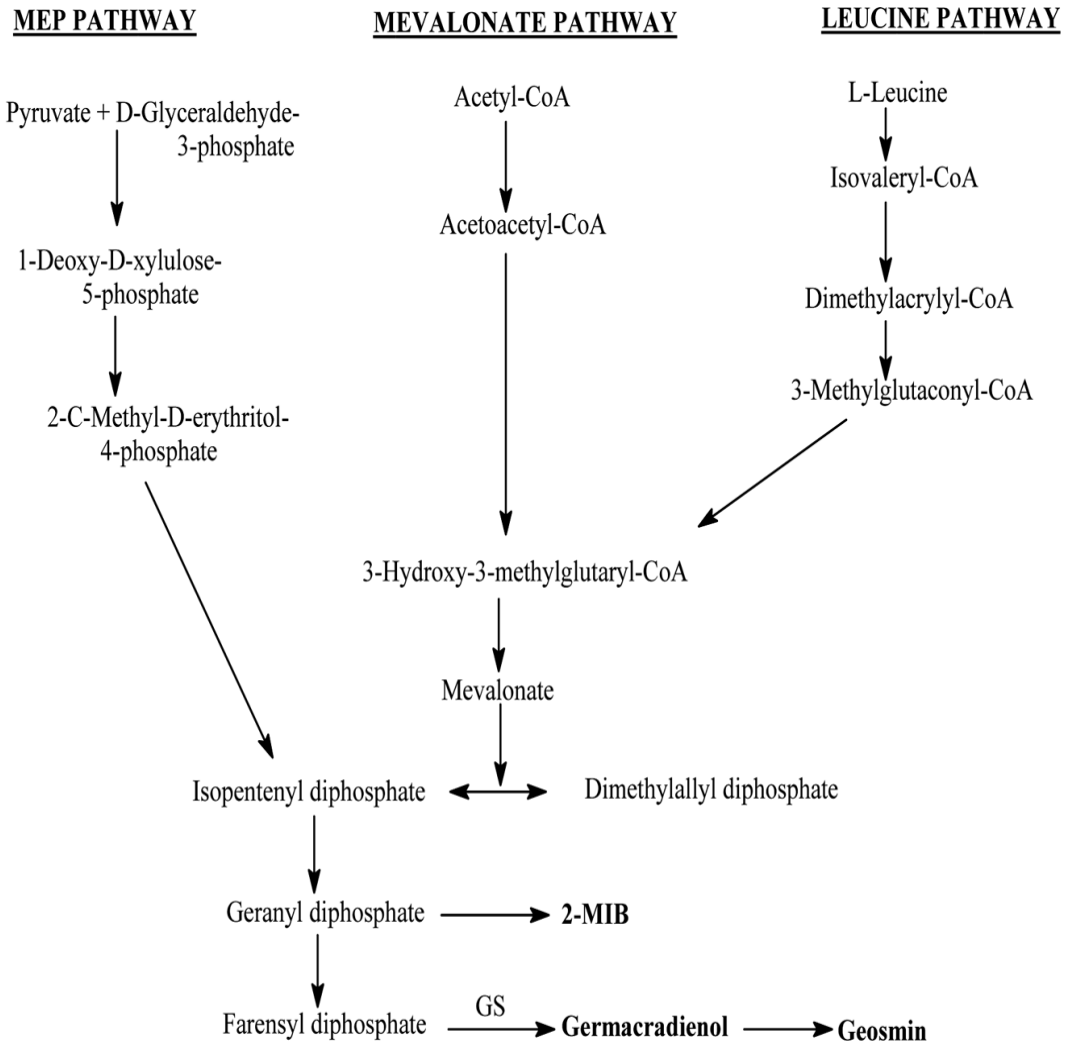


Figure 5: Simplified biosynthetic scheme for the formation of geosmin in streptomycetes and myxobacteria.

1.4.5.2. Genes involved in geosmin biosynthetic pathway in bacteria

Gust *et al.* (2003) reported the involvement of a sesquiterpene synthase domain of a protein encoded by the gene, *SCO6073* (*cyc2*) in geosmin biosynthesis in *Streptomyces coelicolor*. This protein has two sesquiterpene domains but only the amino-terminal sesquiterpene synthase domain of *cyc2* was found essential for geosmin biosynthesis from farnesyl diphosphate. Later, the characterization of a *Streptomyces coelicolor* germacradienol/geosmin synthase was done by Cane and Watt (2003). The *S. coelicolor A3* (*SCO6073*), encodes a protein of 726 amino acids that was showed to catalyze the Mg^{2+}-dependent conversion of farnesyl diphosphate to the germacradienol. In geosmin biosynthesis, the formation of the germacradienol seemed to be the committed step. Cane *et al.* (2006) performed cloning and expression analysis of the *S. avermitilis* gene *SAV2163* (*geoA*) that encodes a germacradienol/geosmin synthase. The *S. avermitilis geoA* is extremely similar to the *S. coelicolor A3* gene. Another gene *spterp13* having 2,199 bp encoding a germacradienol/geosmin synthase was functionally characterized from *Streptomyces peucetius* ATCC 27952. The amino acid sequence of Spterp13 showed 66 % identity with GeoA from *S. avermitilis* and 65 % identity with A3 from *S. coelicolor*, which were reported to produce geosmin (Ghimire *et al.*, 2008). Giglio *et al.*, 2008 reported the characterization of the 1893-bp *npun02003620* gene of *Nostoc punctiforme* PCC 73102 (ATCC 29133) also encoding germacradienol/geosmin synthase. They demonstrated for the first time that geosmin biosynthesis in a model cyanobacterium, *Nostoc punctiforme* PCC 73102 (ATCC 29133) utilized a single enzyme to catalyze the cyclization of farnesyl diphosphate to geosmin. Ludwig *et al.* (2007) amplified two genes *geoA1* and *geoA2* in *Phormidium* sp., by PCR. These two amplified genes were similar to the *cyc2* and *geoA* genes of *Streptomyces* involved in geosmin biosynthesis.

Germacradienol/geosmin synthase is a bifonctional enzyme and catalyze the conversion of farnesyldiphosphate into geosmin in a two-step process. The germacradienol/geosmin synthase catalyzes the Mg^{2+} -dependent conversion of farnesyldiphosphate to a mixture of germacradienol, germacrene D, octalin and geosmin, without involvement of any cosubstrates or redox cofactors (Figure 6). Infact, the Mg^{2+} -dependent cyclization of FPP to germacradienol and germacrene D is catalysed by the recombinant N-terminal half of *S. coelicolor* SCO6073 protein, while the highly homologous C-terminal domain, previously thought to be catalytically silent, catalyzes the Mg^{2+}-dependent conversion of germacradienol to geosmin. Both the N- and C-terminal halves of the *S. coelicolor* SCO6073 protein harbor variants of the canonical aspartate rich domain, with a DDHFLE motif in the N-terminal half and an unusual DDYYP motif in the C-terminal half. Typical NSE motifs: NDLFSYQRE and NDVFSYQKE are also present in both halves. An unusual repeat of the upstream NSE motif NDVLTSRLHQFE is also located in the N-terminal half. (Figure 7) (Jiang *et al.*, 2006; 2007).

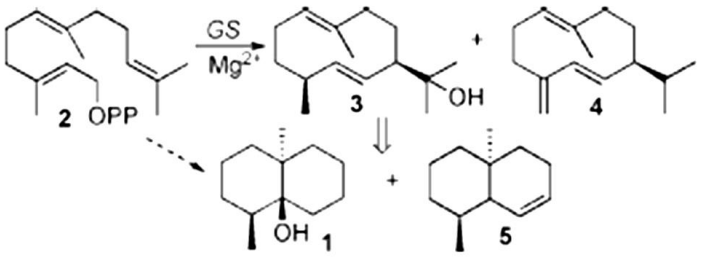


Figure 6. Mechanism of cyclization of FPP (**2**) to Germacradienol (**3**), Germacrene D (**4**), Hydrocarbon **5**, and Geosmin (**1**).

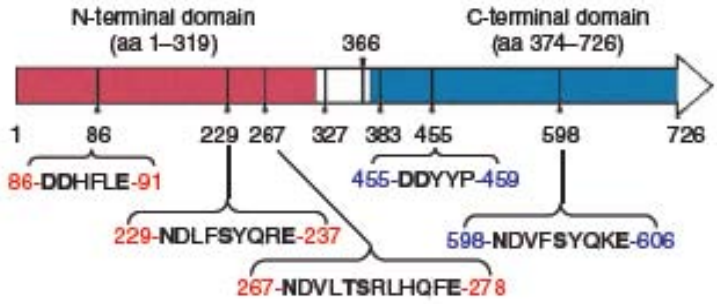


Figure 7: Organization of protein domain and conserved Mg^{2+}-binding motifs in *S. coelicolor* germcradienol-geosmin synthase. The N-terminal domain, corresmonding to amino acids 1-319 is highlighted in red. The C-terminal domain, corresmonding to amino acids 374-726, is shown in blue.

1.4.5.3. Mechanim and stereochemistry of the conversion of farnesyldiphosphate to germacradienol and germacrene D

Intermediate products and catalytic mechanisms have been also documented in several bacterial species (Dickschat *et al.*, 2005; Jiang and Cane, 2008). The profile of volatile compounds emitted by the myxobacterium *Myxococcus xanthus* (strain DK1622) has been investigated by use of a modified closed-loop stripping apparatus (CLSA). One of the major terpenoid compounds produced by this strain was the geosmin. Other terpenoids released by *M. xanthus* were germacradienol, often produced in large amounts, and germacrene D (Dickschat *et al.*, 2005).

Jiang and Cane, 2008 have investigated in detail the mechanism and stereochemistry of the conversion of farnesyldiphophate to germacradienol (3) and germacrene D (4), which is shown to involve the partitioning of a common germacradienyl cation intermediate (6) (Figure 8). A 1,3-hydride shift of the original H-1*si* of FPP results in the formation of germacrene D. The alternative germacradienol formation, which involves competing loss of the H-1*si* proton of FPP (2), can occur by cyclization of 6 to an enzyme-bound, trans-fused

bicyclic intermediate, isolepidozene (7). By proton-initiated ring opening and capture of the resulting homoallyl cation by water, isolepidozene would be converted to germacradienol. The formation of octalin (5) and release of the 2-propanol side chain as acetone is due to proton-initiated cyclization of germacradienol and retro-Prins fragmentation. Then, geosmin is produced by reprotonation of 5 followed by 1, 2-hydride shift of the bridgehead proton into ring B and quenching of the resulting cation by water. The isolation of octalin (5) as a coproduct of incubations of FPP with germacradienol/geosmin synthase gave a support to this model.

Figure 8: Mechanism and stereochemistry of the cyclization of FPP (2) to germacradienol (3), germacrene D (4), octalin (5) and geosmin (1).

Objectives

The objectives of the thesis were:

- *Penicillium expansum* is responsible for geosmin production in wines and grape juices. In order to better understand the production of geosmin by *P. expansum*, we proposed a study of the biosynthesis pathway of the geosmin in *P. expansum*. The objective of our work was the characterization of the genes implicated in the biosynthesis of the geosmin in *P. expansum*.
- To searche genetic biomarkers of the geosmin to manage the presence of geosmin in the wine industry.

Chapter II

Materials and Methods

2.1. Materials

2.1.1. List of products utilised

- Agarose Mol Bio Grade (Promega, Charbonniéres, France)
- Agar (Difco, Fisher Bioblock Scientific, Illkirch, France)
- Acetic acid (Sigma Aldrich, Saint Quentin Fallavier, France)
- Ampicillin (Sigma Aldrich, Saint Quentin Fallavier, France)
- CTAB (Sigma Aldrich, Saint Quentin Fallavier, France)
- Chloroform (Fisher Bioblock Scientific, Illkirch, France)
- Copper sulfate (Fluka, Saint Quentin Fallavier, France)
- Ethanol (Fluka, Saint Quentin Fallavier, France)
- EDTA (Sigma Aldrich, Saint Quentin Fallavier, France)
- Ethidium bromide (Sigma Aldrich, Saint Quentin Fallavier, France)
- Fructose (Fluka, Saint Quentin Fallavier, France)
- Glycerol (Fluka, Saint Quentin Fallavier, France)
- Hygromycin B (Calbiochem, VWR International, Fontenay sous Bois, France)
- Hydrochloric acid (Fluka, Saint Quentin Fallavier, France)
- Iron sulfate (Fluka, Saint Quentin Fallavier, France)
- Isopropanol (Sigma Aldrich, Saint Quentin Fallavier, France)
- LB medium (Luria-Bertani) (Difco, Fisher Bioblock Scientific, Illkirch, France)
- Lysing enzymes (Sigma Aldrich, Saint Quentin Fallavier, France)
- Magnesium sulfate heptahydrat (Fluka, Saint Quentin Fallavier, France)
- Maltose (Fluka, Saint Quentin Fallavier, France)
- Peptone (Fisher Bioblock Scientific, Illkirch, France)
- Potassium phosphate monohydrogen (Fluka, Saint Quentin Fallavier, France)
- Potassium phosphate dihydrogen (Fluka, Saint Quentin Fallavier, France)

- Potassium chloride (Sigma Aldrich, Saint Quentin Fallavier, France)
- Potassium hydroxyde (Sigma Aldrich, Saint Quentin Fallavier, France)
- Polyethylene glycol 6000 (Sigma Aldrich, Saint Quentin Fallavier, France)
- Phenol-chloroform-isoamyl alcohol : 25/24/1 (v/v/v) (MP Biomedicals & Qbiogene,Illkirch, France)
- Restriction enzymes: Ecor1, Not1, Sal1, Sma1. (MP Biomedicals & Q-biogene, Illkirch, France)
- Sodium nitrate (Fluka, Saint Quentin Fallavier, France)
- Sodium chloride (Sigma Aldrich, Saint Quentin Fallavier, France)
- Sodium hydroxyde (Fluka, Saint Quentin Fallavier, France)
- Sucrose (Fluka, Saint Quentin Fallavier, France)
- Sodium dodecyl sulfate (Euromedex, Paris, France)
- Sorbitol (Sigma Aldrich, Saint Quentin Fallavier, France)
- Tween 80 (Fisher Bioblock Scientific, Illkirch, France)
- Tris (Sigma Aldrich, Saint Quentin Fallavier, France)
- Tris-Acetate, EDTA 50X (MP Biomedicals & Qbiogene, Illkirch, France)
- X-Gal (Euromedex, Paris, France)
- Yeast extract (Difco, Fisher Bioblock Scientific, Illkirch, France)
- Ammonium Sulfate (Fluka, Saint Quentin Fallavier, France)
- Ammonium nitrate (Fluka, Saint Quentin Fallavier, France)
- Ammonium chloride (Sigma Aldrich, Saint Quentin Fallavier, France)
- Manganese sulfate (Fluka, Saint Quentin Fallavier, France)
- Malt Extrait (Difco, Fisher Bioblock Scientific, Illkirch, France)
- Potassium chloride (Sigma Aldrich, Saint Quentin Fallavier, France)
- Sodium borate (Fluka, Saint Quentin Fallavier, France)
- Sodium sulfite (Na_2SO_3) (Fluka, Saint Quentin Fallavier, France)

- Tartaric acid (Sigma Aldrich, Saint Quentin Fallavier, France)
- Zinc sulfate (Fluka, Saint Quentin Fallavier, France)

2.1.2. List of Kit utilized

Kits	Producers
Fast DNA SPIN kit	MP Biomedical and Qbiogene, Illkrich, France
EZNA Fungal DNA Miniprep Kit	Biofidel, Vaulex en Velin, France
Purelink Quick Plasmid Miniprep Kit	Invitrogen, Cergy Pontoise, France
QIAquick Gel Extraction Kit	QIAGEN, Courtaboeuf, France
QIAquick Nucleotide Removal Kit	QIAGEN, Courtaboeuf, France
QIAquick PCR Purification Kit	QIAGEN, Courtaboeuf, France
TOPO TA Cloning Kit	Invitrogen, Cergy Pontoise, France
SuperCos 1 Cosmid Vector Kit	Stratagene USA and Canada
Gigapack III gold packaging extract	Stratagene USA and Canada
DNeasy plant Maxi kit	Qiagen, Courtaboeuf, France

2.1.3. Apparatus used

- pH-meter (Corning- EEL modèle 109)
- Autoclave
- Vacuum concentrator (Certomat RB Braun, Unimax 2010, Heidolph, Germany)
- Water bath (Bioblock Scientific, Polystat I ref. 33194 220 V)
- Mixer (Type 418, Braun, Spain)
- Centrifuge (Jouan)
- Micro centrifuge (112, Sigma Aldrich, Saint Quentin Fallavier, France)

- Precision balance (OSI, M-220 D, Drnver Instrument)
- Normal balance: Sartorius B 610 S, (Mettler, type B6C 200, Mettere E Mettler)
- Incubator (Héraeus)
- Microscope (Leica, Leitz DM RB)
- Homogenizer (Ultra Thurax)
- UV-visible spectrophotometer (Philips PU 8600)
- Electrophoresis tank, horizontal system (Embi Tec, San Diego, CA)
- Thermo cycler (Robocycler gradient 96 BioRad)
- Table-top UV transilluminator
- Quantity one analysis software (BioRad, France)

2.1.4. Culture media

2.1.4.1. Yeast Extract Saccharose (YES)

Composition for 1 liter

Saccharose (1M)	:	341 g
Yeast extract	:	20 g
Agar	:	15 g
Distilled Water	:	qsp 1L

Autoclave for 15 min at 121°C

2.1.4.2. Czapek Yeast Extract Agar (CYA)

Preparation of CYA medium needs an advanced preparation of three solutions i.e. solution A, C and Cu+Zn.

Composition for 1 liter

Saccharose	:	30 g
Yeast extract	:	5 g
Solution A	:	50 mL
Solution C	:	50 mL
Solution Cu+Zn	:	1 mL
Agar	:	15 g
Distilled Water	:	qsp 1L

Autoclave for 15 min at 121°C

Solution A (500 mL)

NaNO3	:	20 g
KCl	:	5 g
MgSO4.7 H2O	:	5 g
FeSO4.7 H2O	:	0,1 g
Distilled Water	:	qsp 1L

Solution C (500 mL)

K2HPO4	:	10 g
Distilled Water	:	qsp 500 mL

Solution Cu+Zn (100 mL)

ZnSO4, 7H2O	:	1 g
CuSO4, 5H2O	:	0,5 g
Distilled Water	:	qsp 500 mL

2.1.4.3. LB Broth

Composition for 1 liter

NaCl	:	10 g
Trypton	:	10 g
Yeast extract	:	5 g

Adjust a pH 7.0 with 5 N NaOH

Add distilled water to adjust final volume to 1 liter

Autoclave for 15 min at 121°C.

2.1.5. Oligonucleotides primers used

Primer name	Sequence (5' -3')
mhsF	CGAAATTCTGCTGGAAAGCG
mhsR	ATTGGCTTTTCCCGTTCACG
hphF	GAATTCAGCGAGAGCCTGAC
hphR	ACATTGTTGGAGCCGAAATC
M13F	ACGTTGTAAAACGACGGCCAG
M13R	CAGGAAACAGCTATGACCATG

2.2. Methodology

2.2.1. Preparation of inoculum and their conservation

On a Petri plate containing YES medium, a spore suspension (10^6 spores / mL) of *Penicillium expansum* was spread. 5 to 10 mL of 0.01 % Tween 80 solution was spread on each plate after 10 days of incubation at 28 °C. The spores were scratched with a sterile blade and filtered with a gaze. The counting of the spores was done through Thoma Bright line counting chamber.

The spore solutions (glycerol 50% - spores suspension (50:50 (v/v))) of the fungal strain were made in cryogenic tubes of 1.8 mL (Nalgene, Fisher Bioblock Scientific, Illkirch, France) and conserved at -20 °C.

2.2.2. Fungal nucleic acid extraction

a. Preparation of fungal material

Inoculation of spores suspension (final concentration of 10^6 spores / mL) was done in an Erlenmeyer flask (250 mL) containing liquid YES medium (100 mL) and incubated at 28 °C without agitation for 3 days. Then, filtration of liquid culture was performed through a nitrocellulose membrane of 0.45µm. The recovered fungal material was grounded in liquid nitrogen and stored at -80 °C for subsequent extraction of nucleic acids.

b. Extraction of genomic DNA

The DNA molecules were released by lyses of the fungal cell wall and cell membrane. The processing of RNA and proteins were performed by the RNase A and proteinase K (Promega, Charbonnieres, France) respectively. The genomic DNA was re-suspended in ultra high quality water. The following three methods were used to extract genomic DNA.

I. Extraction of high molecular weight DNA

Mycelium was rinsed several times with distilled water, excess water was removed and one gram of mycelium was grounded by mortar and pestle after being froze in liquid nitrogen. The extraction of DNA was performed in a 250 mL Erlenmayer flask with 25 ml of TSE buffer (150 mM NaCl, 100 mM EDTA, 50 mM Tris HCl) containing 2 % SDS and 0.2 volumes of toluene. The samples were centrifuged at 2000 x g for 15 minutes after incubation for 72 hours at room temperature with shaking (rotary shaker at about 1 cycle per second) and then supernatant was collected. The classical phenol-chloroform technique, involving extraction through 1 volume of phenol, followed by 1 volume of phenol – 1

volume chloroform and then by a final volume of chloroform was used for the purification of the DNA by extraction. After phenol chloroform extraction, 0.6 volume of isopropanol was used to precipitate (in the form of a clot) the DNA in the supernatant. The DNA clot was removed with the help of a sterile Pasture pipette, rinsed gently with 70% ethanol, dried in a desiccator and dissolved in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0).

II. CTAB method

A lyses solution or extraction buffer with composition as: CTAB (1%), EDTA (pH 8, 20 mM), NaCl (1.4M), Tris-HCl (pH 8, 100mM) and ultra pure H_2O to adjust the volume, was prepared to extract the DNA. The filtration of the extraction buffer was performed through a 0.22 µm filter (Millipore). The mycelium was grounded in liquid nitrogen. Put about 300 mg of grounded mycelium in an Eppendorf tube, added 700 µL of extraction buffer in it and mixed vigorously, then incubated at 50 °C for 10 minutes and finally transferred to 4 °C for one hour. Added equal volume of phenol-chloroform-isoamyl alcohol (v / v) and vortexed for about 30 seconds to obtain an emulsion. A centrifugation was performed for 15 min at 13,000 rpm and then, the upper phase was collected in a new Eppendorf tube. Then, equal volume of chloroform (v / v) was added to remove residual phenol. A centrifugation was again performed for 5 minutes at 13,000 rpm after vortexing the sample. The upper phase was transferred to a new Eppendorf tube. To precipitate the DNA, 0.7 volume of isopropanol was added and mixed thoroughly. A centrifugation for 10 minutes at 13,000 rpm was performed to have the DNA in a pellet. Then, washed the DNA with about 500 µl of 70 % (v / v) ethanol to remove salts by centrifugation for 5 minutes at 13,000 rpm. Air dried the pellet in the Eppendorf tube and re-suspended in 50 µl of ultra pure sterile water.

The optical density (OD) of the DNA was measured in a spectrophotometer to check the quantity and quality of the extracted DNA. Finally, a horizontal agarose gel

electrophoresis was performed to visualize the DNA.

III. DNA extraction by quick method

To a 1.5 mL Eppendorf tube containing 500 µL of lyses buffer (400 mM Tris HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 1 % sodium dodecyl sulfate), a small quantity of mycelium was taken with the help of a sterile pipette tip and was grounded with the same sterile pipette tip. The tube was incubated for 10 minutes at room temperature. Then, added 150 µL of potassium acetate buffer (composition for a final volume of 100 mL is; 5M Potassium acetate 60 mL, Glacial acetic acid 11.5 mL and distilled water 28.5 mL) into the tube, vortexed briefly and centrifuged for 1 minute at 12,000 rpm. The clear supernatant was transferred to another tube and equal volume of isopropanol was added into it. A centrifugation was performed for 2 minutes at 12,000 rpm after mixing by inversion. Discarded the supernatant and washed the pellet with 300 µL of 70 % (v / v) ethanol. A centrifugation was performed for 1 minute at 12,000 rpm. The supernatant was discarded and the DNA was resuspended in 50 µL of sterile water.

c. DNA quantification

Optical density (OD) the DNA was measured in a quartz vessel at two different wavelengths i.e. at 260 nm, the wavelength of nucleic acids absorption and at 280 nm, the wavelength of proteins absorption. The ratio of OD (OD260 nm/OD280 nm) is a mean to check the purity of DNA samples. There could be a contamination of proteins in the sample if this ratio is less than 1.6 and there could be a contamination of RNAs if it exceeds 1.9.

The following formula gives the amount of DNA in the sample:

[DNA] = OD260 nm × 50* × dilution factor

* 1 unit of OD 260 nm = 50 µg / mL DNA

2.2.3. Horizontal agarose gel electrophoresis

Nucleic acids move through the pores in the agarose gel from the cathode to the

anode due to the electric field present in the horizontal electrophoresis tank. The separation of nucleic acids is based on its size and congestion level, concentration of agarose gel, voltage and ionic strength of the buffer.

a. Gel preparation and migration

1X TAE: Tris-acetate, EDTA (ethylene diamine tetra-acetic acid) buffer was used both for the preparation and the migration of the gel. The salt in the buffer permitted the conduction of the electric field in the horizontal electrophoresis tank. A 0.8 % to 2 % agarose gel was used. The powdered agarose was added in 1X TAE buffer, heated in a microwave till to become a homogeneous solution, let it be cooled a bit and poured into the horizontal tank. Samples were weighed down by loading buffer "Blue / Orange Loading Dye, 6X (Promega)" and were deposited in the wells of agarose gel submerged in 1X TAE buffer. To estimate size of different fragments of nucleic acid, the size marker: 1kb DNA Ladder (Promega) was used as a reference.

b. Visualisation of the gel after electrophoresis

Let the gel be submerged in a tray containing 1X TAE buffer + ethidium bromide (BET) at a final concentration of 0.5 ug / ml after migration. BET is a mutagen which inters to fix itself between the bases of nucleic acids. It allows the visualization of the bands of nucleic acids in its fluorescence under UV (254 nm). The use of BET should be done with great caution as it is highly carcinogenic. Twenty minutes after submersion in solution of BET, the gel was analyzed by using an automated trans-illuminator (Quantity one 3-D analyzer, BIO-RAD).

2.2.4. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a technique to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of a particular DNA sequence. The three steps of a PCR cycle were conducted at specific

temperatures in a thermocycler (BIO-RAD Robocycler gradient 96), which is an automatic and programmable machine allowing the shift of one temperature to another and equally repeating the cycle.

Protocol

A PCR kit supplied by MP Biomedicals was utilized to prepare the reaction mix. This kit is composed of an enzyme i.e. Taq DNA polymerase, dNTPs mix and 10 X PCR buffer with $MgCl_2$. The reaction was composed of:

Primer 1 (10 µM)	:	0.5µl
Primer 2 (10 µM)	:	0.5µl
dNTPs mix (10 mM each)	:	0.5µl
10 X PCR buffer with MgCl2	:	2.5µl
Taq DNA polymerase 5U/µl	:	0,2 unit
DNA	:	~ 100 ng
Sterilized H2O	:	qsp 25 µl

The PCR reaction was programmed as follow:

First step : Initial denaturation at 94 °C for 4 min.

Second step: 30-35 cycles

- Denaturation at 94 °C for 45 sec.
- Hybridation at T[1] °C for 45 sec.
- Elongation at 72 °C for t[2] min.

Third step: - Final elongation at 72 °C for 10 min.

[1] T depends on the primer melting temperature

[2] t depends on the size of the fragment to amplify

2.2.5. Cloning

a. Cloning principle

The cloning of a DNA fragment consists of its insertion in a DNA vector: plasmid in our case, then the recombinant vector is introduced into a host cell (*Escherichia coli*) by thermal shock, which is then spread on Petri dishes containing solid LB agar medium. The bacteria will form a colony of identical cells containing the DNA fragment inserted in the starting plasmid vector by dividing themselves.

b. Preparation of Petri dishes containing LB agar

Two Petri dishes containing LB agar (LB: 25 g / L; Agar: 15 g / L) medium supplemented with 50 µg / mL of ampicillin were prepared and placed in the incubator at 37 °C for 30 min. On each plate, 40 µl of X-gal (5-Bromo-4-chloro-3-indolyl-b-D-galactopyranoside) was equally spread and left the plates at 37 °C for 30 minutes before use.

c. Cloning Protocol

The TOPO TA Cloning kit (Invitrogen) was used was for cloning. The reactants supplied with the kit include:

- TOPO-pCR2.1 vector

Competent cells: these are the *Escherichia coli* (TOP10F' One Shot *E. coli*) cells.

- Saline solution (1.2 m NaCl, 0.06 M $MgCl_2$)

SOC medium (2% Tryptone, 0.5% Yeast Extract, 0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose)

d. ligation

In order to perform the ligation, 1 µl of TOPO-pCR2.1 vector, 4 µl of DNA fragment (PCR product or gel purified DNA fragment) and 1 µl of saline solution were added in a sterile 0.2 mL Eppendorf tube. Mixed gently and put at room temperature for 30

minutes.

e. Transformation

The tube containing ligation mixture was put on the ice after giving a little spin through a table top centrifugation machine. Then, removed the competent cells from - 80 ° C and put them on ice till thawed. Transferred 4 µl of ligation mixture into the tube of competent cells, mixed by gentle shaking and then placed on ice for 30 minutes. The tube was placed for 40 seconds in a water bath at 42 ° C (without stirring) to produce a thermal shock and then, immediately transferred to ice for 5 minutes. Added 250µl of SOC medium into the tube at room temperature and then incubated the tube at 37 °C under agitation (200 rpm) for 1h. 20 µl and 40 µl of the transformation mixture were spread independently on two LB agar Petri dishes containing ampicillin at concentration of 50 µg / mL. Then, the plates were incubated overnight (16 to 24 hours) at 37 °C.

f. Analysis and conservation of clones

After incubation, two types of clones were present on the Petri dishes. The white clones (10 clones) were analyzed by PCR using the primer pair *M13*F / *M13*R. Then, cultures of the positive clones were made in liquid LB medium containing ampicillin at concentration of 50 µg / mL to perform mini preparation. Conserved the recombinant clones in glycerol at a final concentration of 25 %, in cryogenic tubes of 1.8 mL (Nalgene, Fisher) and stored at -20 °C.

e. Mini preparation of plasmid DNA

Mini preparation of plasmid DNA was performed by using a kit (Purelink Quick Plasmid Miniprep Kit, Invitrogen) according to the protocol described by the supplier.

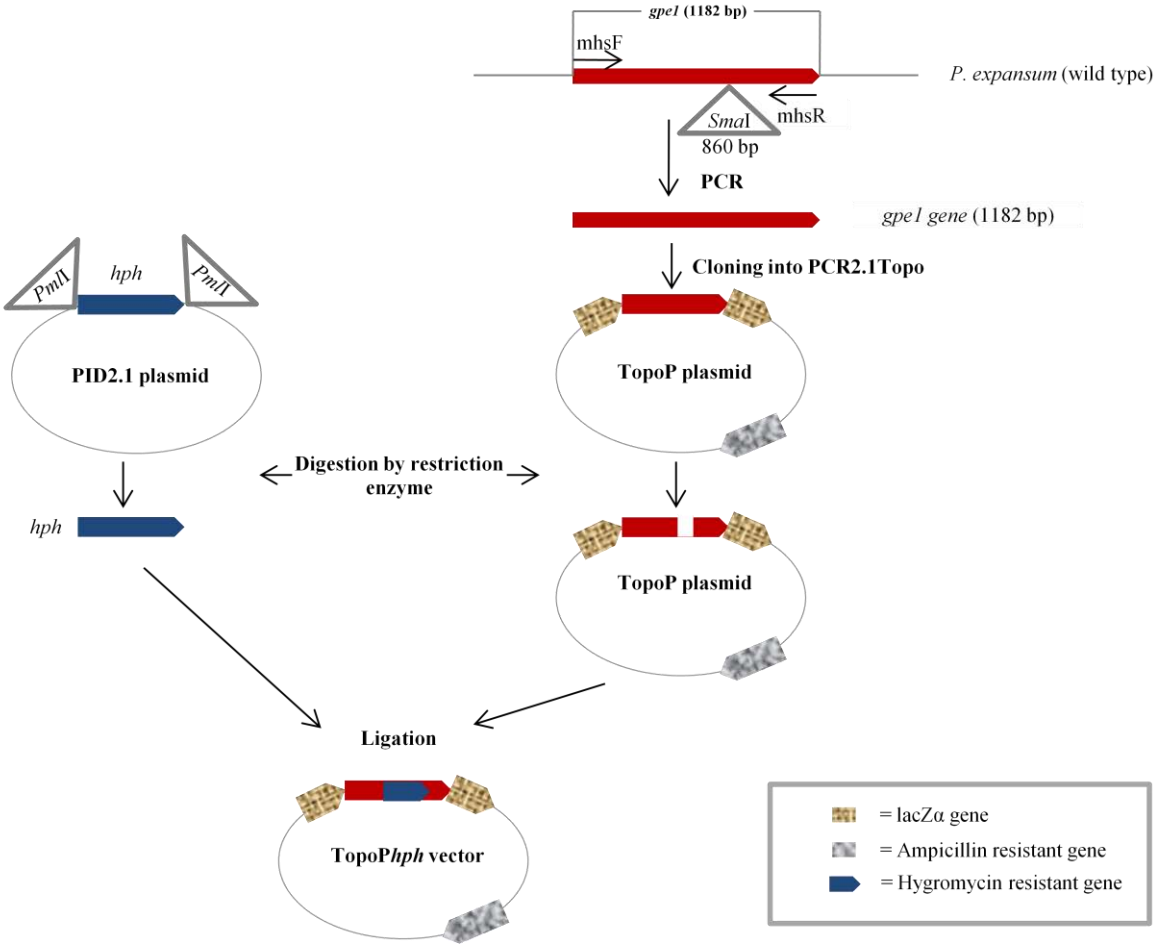
2.2.6. Transformation vectors formation and transformation of *P.expansum*

a. Formation of transformation vector

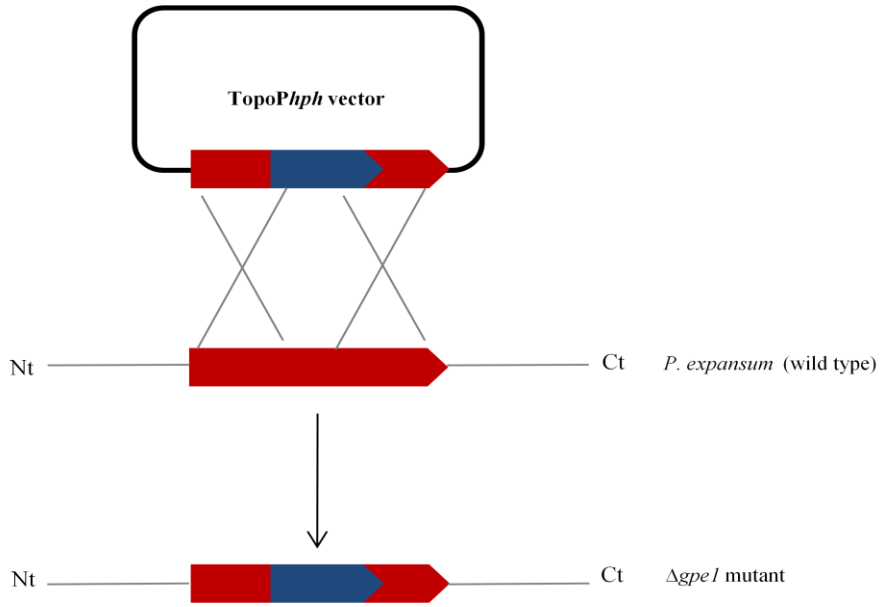
A 1182 bp fragment of the gene of interest was cloned into the plasmid pCR2.1-

TOPO. Besides the hygromycin B phosphotransferase (*hph*) cassette of resistance from *Escherichia coli* was generated from plasmid pID2.1 (Tang *et al.*, 1992) and was inserted into the plasmid pCR2.1-TOPO 1.2 kb fragment thanks to the *Sma*1 digest to form the transformation vector (Figure 9). Performed the final transformation of *P. expansum* by fusion of transformation vector with protoplasts of *P. expansum*.

a.



b.



c.

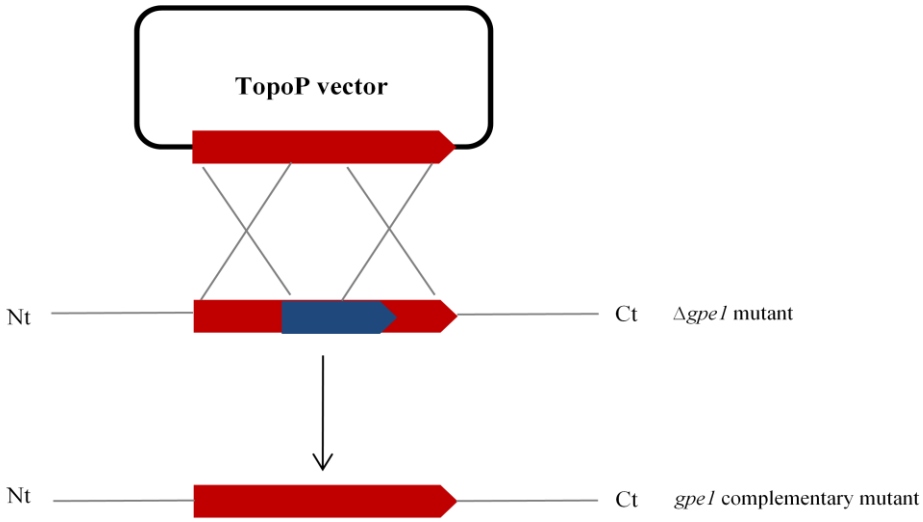


Figure 9. Schematic representation of transformation vector formation and *gpe1* gene disruption. (a) Using primer pair *mhsF*/*mhsR*, 1182 bp *gpe1* gene containing *Sma*I restriction site (indicated by triangle) was amplified. PCR product was cloned into the PCR2.1–Topo plasmid to generate the plasmid TopoP. The PID2.1 plasmid vector was restricted with the restriction enzyme *Pml*I (indicated by triangle) to obtain *hph* cassette

(1032 bp). The plasmid TopoP was restricted with *Sac*1 and ligated with *hph* cassette to generate the TopoP*hph* transformation vector. (b) Protoplasts of *P. expansum* wild type was prepared and *gpe1* gene was disrupted using the TopoP*hph* vector to obtain *Δgpe1* mutants. (c) Protoplasts of *gpe1* mutant was prepared and *gpe1* gene was brought in its original form using the TopoP vector to obtain *gpe1* complementary mutants.

b. Formation of protoplasts

4 x 10^6 conidia / mL of the wild type strain or mutant strain were inoculated in a liquid YES medium for 15 hours until the germination of spores (microscopic verification) at 28 °C under agitation (125 rpm) for the preparation of protoplasts. Recovered the newly germinated mycelium by filtration on the sterilized muslin paper (miracloth, Calbiochem) and then washed with 200 mL of solution 1 (0.6M $MgSO_4$). Dried the muslin paper containing mycelium between sterile paper towels and weighed the dried mycelium in a sterile Petri dish. Then, transferred the mycelium into a sterilized 15 mL tube and re-suspended in 5 mL of solution 2 (1.2 M $MgSO_4$ in 10mM Na_2HPO_4/NaH_2PO_4, pH 5.8). Add lyses enzymes and then placed on ice for 5 min. The preparation was then incubated at 30 °C under shaking at100 rpm for one hour. The formations of protoplasts were confirmed by observation under microscope.

NB: the final concentration of mycelium is 1 g / 10 mL and that of the lyses enzymes is 40 mg / mL.

c. Purification of protoplasts

One volume of separation buffer A (0.6 M sorbitol, 100 mM Tris-Cl, pH 7.0) was added in the protoplasts suspension and centrifuged at 3000 rpm for 15 minutes. Recovered protoplasts from the interphase and transferred into a new falcon tube. The volume was completed to 10 mL by the addition of separation buffer B (1.2 mM sorbitol, 10 mM Tris-HCl, pH 7.5). Then, a centrifugation at 2600 rpm for 10 minutes was performed to collect

the protoplasts in the form of a pellet. A second wash was performed with 10 mL of separation buffer B. Finally, the protoplasts were resuspended in 0.2 mL of solution 7 (1.0 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5). Determined the concentration of protoplasts and then, adjusted the concentration to 2 x 10^6 protoplasts / mL with solution 7.

d. Transformation

The digestion of the prepared transformation vector was performed by *EcoR*1 restriction enzyme and then purified the plasmid DNA with ethanol precipitation. To a sterilized Eppendorf tube, added about 3 µg of plasmid DNA, then, 150 µl of protoplasts solution (2 x 10^6 protoplasts / mL) was added in the tube and put the tube on ice for 20 min after mixing gently. Then, 500 µl of solution 8 (60% PEG, 10mM $CaCl_2$, 10mM Tris-HCl) were added into the tube and placed at room temperature for 20 minutes. 500 µl of solution 7 was added to the mixture and was then gently suspended in 30 mL of overlay medium (0.3 % agar YES). Then, distributed this mixture suspended in overlay medium on 10 Petri dishes containing solid YES medium. Incubated all the plates at room temperature for one day. For mutants, about 3ml of 0.3 % agar YES medium containing hygromycin B at a final concentration of 150 µg / mL was added to each Petri dish. The dishes were then incubated at 28 °C for 4 days until the appearance of colonies. The colonies were then sub-cultured on fresh solid YES medium supplemented with hygromycin B at a concentration of 150 µg / mL on Petri dishes.

The initial screening of the genetically complemented mutants without selection cassette was performed by growing them on Petri dishes containing YES medium. After 48 hours of incubation at room temperature, each of the individual grown was divided into two halves. Transferred the one part of the divided colony on to a Petri dish containing YES medium without hygromycin and the other part on a dish containing YES medium supplemented with hygromycin at concentration of 150 µg / mL. The successfully

complemented mutants lost their efficiency to grow against hygromycin.

The positive mutants and complemented mutants which proved positive by initial screening were selected for further screening through PCR.

2.2.7. Confirmation of gene disruption and analyses of mutants

The transformation efficiency was analyzed by several PCR tests utilizing several *hph* specific and gene of interest specific primers (to confirm disruption of the corresponding gene in the genome of subject fungal strain).

2.2.8. Quantification of Geosmin production

The production of geosmin was quantified from 10 days old culture of *P. expansum* wild type, Δ*gpe1* mutant and *gpe1* complementary mutant strain grown in Petri dishes containing YES agar medium. We put all the mycelium along with medium in a tube after cutting into small pieces with a sterile surgical blade. 10 mL of 20 % ethanol was 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 hour. Then, filtered samples were sent to Exact Laboratory at Macon, France for quantification of geosmin production. They quantified the geosmin by gas chromatography–mass spectrometry (GC–MS) with limit of quantification as 10 ng / L. Here is the methodology used for geosmin quantification.

SPME extraction of samples of wine / hydroalcoholic macerate:

5 ml of wine / hydroalcoholic macerate was put in a glass vial of 20 ml, saturated with 3 g of sodium chloride and diluted to 50 % in HPLC grade water previously acidified to pH = 3. Then, 100 ng / L geosmin d5 (internal standard) was also added to mixture. The vial was crimped using a magnetic capsule. The adsorption was carried out on the SPME headspace of the above mixture. It was performed on a fiber of poly-dimethylsiloxane (PDMS) 100µm thick, at 40 ° C for 30 minutes with stirring.

Chromatographic analysis:

After adsorption of the headspace of the sample, the volatiles were desorbed for 10 minutes in the injector of a gas chromatograph VARIAN 3900 coupled to a mass spectrometer ion trap VARIAN 2100. The injection was performed in splitless mode at 260 ° C and separation of volatile compounds was carried out on a capillary column DB5-MS (Varian) of dimensions: length 30 m, internal diameter 0.25 mm, film thickness 0.25 microns. The programming of oven temperature was as follows: 50 ° C – 1 minute; 8 ° / min up to 170 ° C, 25 ° / min up to 280 ° C, 280 ° C – 4; 60 minutes.

The analysis of geosmin and geosmin d5 was performed in MS / MS mode by selective fragmentation of ions 112 and 114 respectively. The quantification was performed on the ions son 97 (geosmin) and 99 (geosmin d5).

2.2.8. Data analyses

The deduced amino acid sequence was determined using the http://www.expasy.org/tools/dna.html site while BLAST 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.

Chapter III

Results and Discussions

3. Results and Discussions

3.1. Whether cytochrome P450 monooxygenase genes can be involved in geosmin production?

3.1.1. Bioinformatics analysis to identify germacradienol/geosmin synthase in *Penicillium*

The biosynthesis pathway of geosmin has been well characterized in bacteria particularly in the genus *Streptomyces*. A bifunctional enzyme germacradienol/geosmin synthase has been found involved in biosynthesis of geosmin in bacteria, in which the N-terminal domain of the protein converts farnesyl diphosphate (FPP) which is the immediate precursor of cyclic sesquiterpenes, into germacradienol and germacrene D, while the C-terminal domain catalyzes the transformation of germacradiennol to geosmin (Jiang *et al.*, 2007). In bacteria, four genes named as: *Streptomyces coelicolor A3* (SCO 6063), *Streptomyces avermitilis geoA* (SAV 2163), *Streptomyces peucetius strept13* (ATCC 27952) and *Nostoc punctiforme pcc 73102* (ATCC 29133) encoding germacradienol/geosmin synthase have been identified and characterized (Cane *et al.*, 2006; Ghimire *et al.*, 2008; Jiang *et al.*, 2006; Giglio *et al.*, 2008).

According to our knowledge no gene encoding germacradienol/geosmin synthase has been characterized in the eukaryotes until today. In order to determine the presence of germacradienol/geosmin synthase in the databases of the genus *Penicillium*, we undertook a bioinformatics search. A BLAST search with the protein sequence of the *S. peucetius strept13* (ATCC 27952) as a query did not show any gene having homology with the genes encoding germacradienol/geosmin synthase, in the genus *Penicillium* (Figure 10). So, no presence of germacradienol/geosmin synthase in the genus *Penicillium* suggest the presence of a different biosynthetic pathway of geosmin in the *P. expansum*.

XP_002149866.1 pentalenene synthase, putative [Penicillium marneffei ATCC 18224] >gb|EEA21257.1| pentalenene synthase, putative [Penicillium marneffei ATCC 18224]

Q03471.1 RecName: Full=Aristolochene synthase; Short=AS; AltName: Full=Sesquiterpene cyclase >gb|AAA33694.1| aristolochene synthase [Penicillium roqueforti]

XP_002557473.1 Pc12g06310 [Penicillium chrysogenum Wisconsin 54-1255] >emb|CAP80258.1| Pc12g06310 [Penicillium chrysogenum Wisconsin 54-1255]

XP_002563575.1 Pc20g10860 [Penicillium chrysogenum Wisconsin 54-1255] >emb|CAP86415.1| Pc20g10860 [Penicillium chrysogenum Wisconsin 54-1255]

1DI1_A Chain A, Crystal Structure Of Aristolochene Synthase From Penicillium Roqueforti >pdb|1DI1|B Chain B, Crystal Structure Of Aristolochene Synthase From Penicillium Roqueforti >pdb|1DGP|A Chain A, Aristolochene Synthase Farnesol Complex >pdb|1DGP|B Chain B, Aristolochene Synthase Farnesol Complex

XP_002560477.1 Pc16g00550 [Penicillium chrysogenum Wisconsin 54-1255] >emb|CAP92725.1| Pc16g00550 [Penicillium chrysogenum Wisconsin 54-1255]

XP_002563784.1 Pc20g13020 [Penicillium chrysogenum Wisconsin 54-1255] >emb|CAP86631.1| Pc20g13020 [Penicillium chrysogenum Wisconsin 54-1255]

XP_002151452.1 heat shock protein/chaperonin HSP78, putative [Penicillium marneffei ATCC 18224] >gb|EEA20452.1| heat shock protein/chaperonin HSP78, putative [Penicillium marneffei ATCC 18224]

XP_002562449.1 Pc18g06220 [Penicillium chrysogenum Wisconsin 54-1255] >emb|CAP94846.1| Pc18g06220 [Penicillium chrysogenum Wisconsin 54-1255]

XP_002150361.1 hypothetical protein PMAA_055480 [Penicillium marneffei ATCC 18224] >gb|EEA21752.1| hypothetical protein PMAA_055480 [Penicillium marneffei ATCC 18224]

XP_002559307.1 Pc13g08820 [Penicillium chrysogenum Wisconsin 54-1255] >emb|CAP91951.1| Pc13g08820 [Penicillium chrysogenum Wisconsin 54-1255]

XP_002567165.1 Pc21g00930 [Penicillium chrysogenum Wisconsin 54-1255] >emb|CAP94990.1| Pc21g00930 [Penicillium chrysogenum Wisconsin 54-1255]

XP_002563154.1 Pc20g06250 [Penicillium chrysogenum Wisconsin 54-1255] >emb|CAP85954.1| Pc20g06250 [Penicillium chrysogenum Wisconsin 54-1255]

XP_002560372.1 Pc15g01520 [Penicillium chrysogenum Wisconsin 54-1255] >emb|CAP83038.1| Pc15g01520 [Penicillium chrysogenum Wisconsin 54-1255]

Figure 10. A BLAST search with the protein sequence of the *S. peucetius strept13* (ATCC 27952) showing absence of the genes encoding germacradienol/geosmin synthase, in the genus *Penicillium*.

3.1.2. Role of P450s in terpenes biosynthesis

No evidence of the presence of germacradienol/geosmin synthase in the *P. expansum* force to think that some other enzymes might be involved in the geosmin biosynthesis. Geosmin structure and the presence of one hydroxyl group (Figure 4) may lead to the role of other enzymes in geosmin production. These enzymes could also be cytochrome P450 monoxygenases as one cytochrome P450 (CYP180A1) has already been predicted to be involved in the biosynthesis of geosmin (Lamb *et al.*, 2003). Trichothecenes are a group of sesquiterpenes produced by *Fusarium* species. Four cytochrome P450 enzymes have been found involved in tichothecenes biosynthesis. Three P450 enzymes: Tri11 (CYP65 family), Tri13 (CYP526 family) and Tri1 (CYP68 family) catalyze the hydroxylation reactions in the biosynthesis pathway at carbons C-15, C-4 and C-8 respectively, while the fourth P450

(Tri4, CYP58 family) is responsible for four consecutive early oxygenation steps that allow trichothecene skeleton formation (Kimura *et al.*, 2007).

In cyanobacteria, terpene synthases are part of an apparent minicluster that includes a P450 and a putative hybrid two-component protein located downstream of the terpene synthases. Coexpression of P450 genes with their adjacent located terpene synthase genes in *E. coli* demonstrated that the P450 oxygenates the terpene product germacrene A (Agger *et al.*, 2008). A cytochrome P450, CYP170A1 has also been found showing terpenenoid synthase activity to generate farnesene isomers from farnesyl diphosphate (FPP) (Zhao *et al.*, 2009).

3.1.3. Amplification *of P. expansum* P450 (*gpe1*) gene sequence by PCR

It has been already a known fact that *P. expansum* is one of the producers of geosmin and this earthy-musty compound belongs to the terpene family. On the other hand, cytochrome P450s enzymes have been found involved in metabolic pathways of terpenes and their derivatives (Nelson *et al.*, 1993; Werck-Reichhart and Feyereisen, 2000; Bernhardt, 2006). The two cytochrome P450 gene fragments i.e. *p450-1* and *p450-2* have been proposed to be involved in patulin biosynthesis in *P. expansum* (White *et al.*, 2006). Later, the functional characterization of the two cytochrome P450 genes i.e. CYP619C2 and CYP619C3 involved in the biosynthesis of patulin in *Aspergillus clavatus* have also been reported (Artigot *et al.*, 2009). We did an alignment of the deduced amino acid sequences of the cytochrome P450 gene fragments i.e. *p450-1* and *p450-2* of *P. expansum* with the CYP619C2 and CYP619C3 (cytochrome P450s) involved in patulin biosynthesis in *Aspergillus clavatus*. This alignment showed a very weak identity (Figure 11) among the P450 genes of *P. expansum* and the P450 genes of *Aspergillus clavatus* needed for patulin synthesis. The results of this alignment suggested another role of *p450-1 and p450-2* in *P. expansum* rather than the involvement in patulin synthesis. So, keeping in view the

production of geosmin by the *P. expansum*, the belonging of the geosmin to the terpene family and involvement of cytochrome P450s genes in the biosynthesis pathway of terpenes, we were wondering about the involvement of *p450-1* and *p450-2* in the biosynthesis of geosmin. Therefore, we performed the alignments of *p450-1 and p450-2* gene fragment of *P. expansum* with a gene involved in terpene biosynthesis.

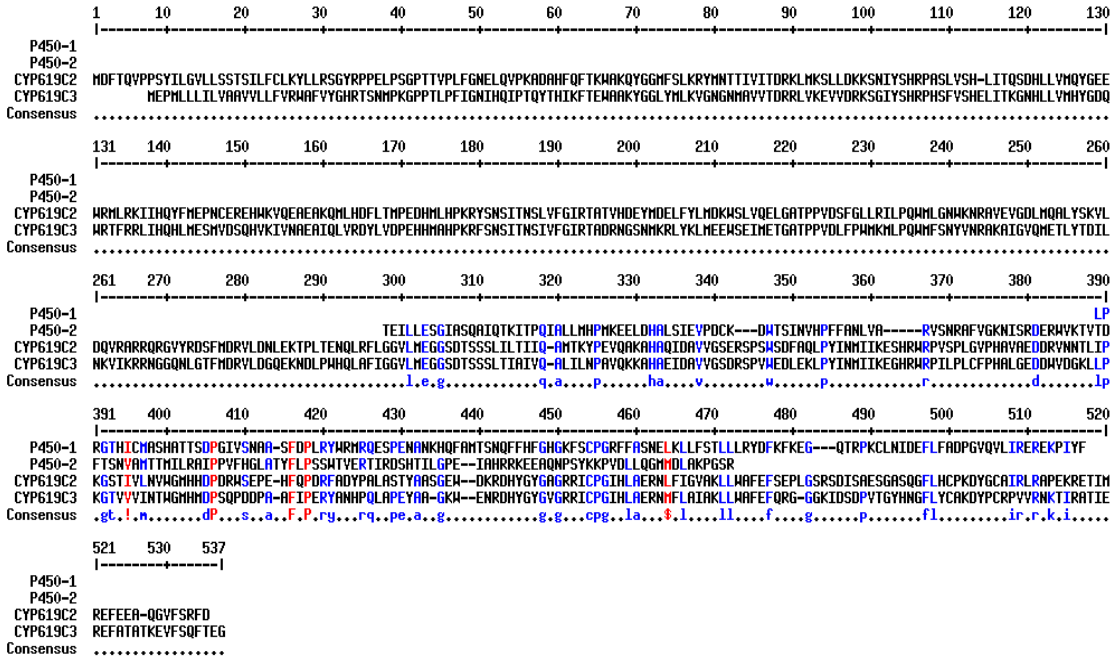
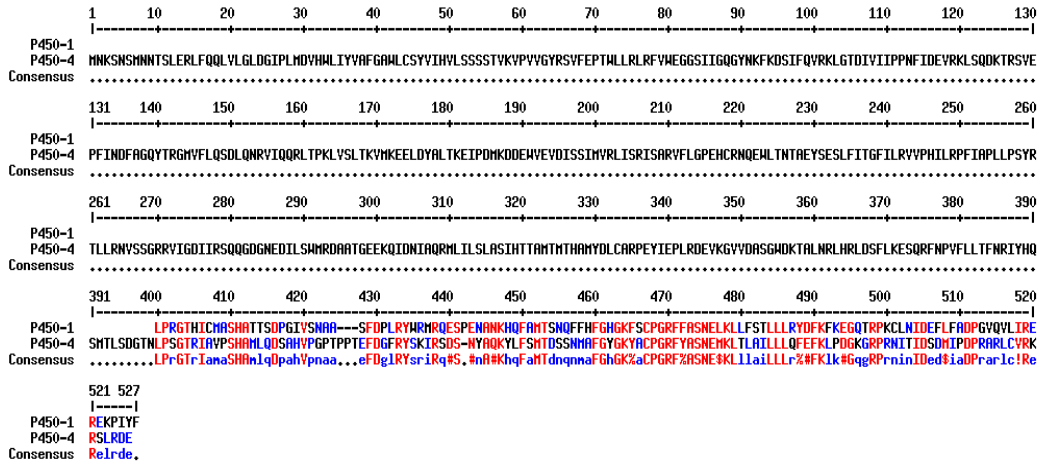


Figure 11. Alignment of the deduced amino acid sequence of *p450-1* (Accession No. DQ084389) and *p450-2* (Accession No. DQ084390) of *Penicillium expansum* with *CYP619C2* (Accession No. EU678353) and *CYP619C3* (Accession No. EU678354) of *Aspergillus clavatus* involved in patulin biosynthesis.

These alignments showed an identity of about 40 % of *p450-1* and *p450-2* with the terpene synthase (Figure 12a, b). The results of these alignments also lead to a different role of *p450-1* and *p450-2* in *P. expansum* contrary to involvement in patulin biosynthesis. Moreover, the partial sequences of *p450-1* and *p450-2* seemed to match with different parts of the same protein. Therefore, we made the hypothesis that the two previously cloned DNA fragments belong to the same gene. In order to test this hypothesis, we designed two primers

i.e. forward primer *mhsF* corresponding to the 5' end of *p450-2* and reverse primer *mhsR* corresponding to the 3' end of *p450-1*. A PCR from this pair of primers was conducted on *P. expansum* (strain M2230). This PCR resulted into the amplification of a single *p450* gene (*gpe1*) fragment of ~1.2 kb (Figure 13). By sequencing, we got the complete nucleotide sequence of the *gpe1* gene consisting of 1182 bp.

a.



b

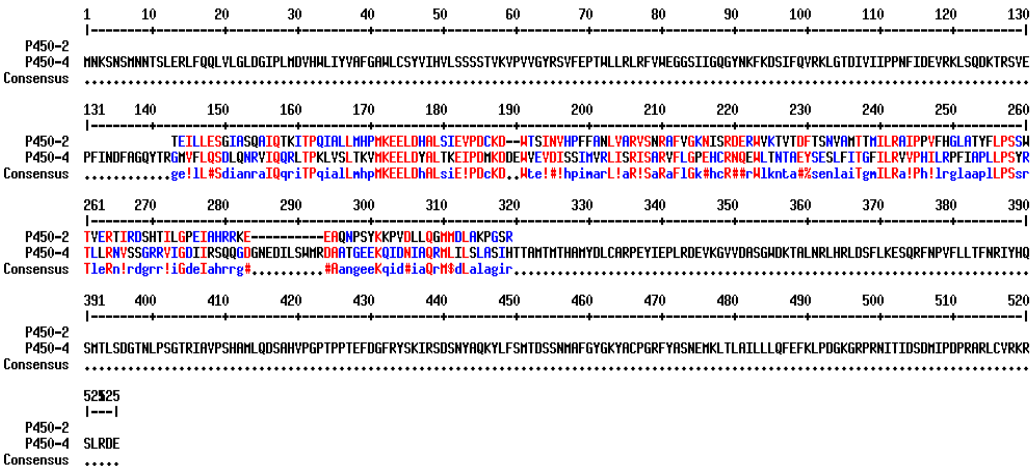


Figure 12: a. Alignment of the deduced amino acid sequence of *p450-1* (Accession No. DQ084389) of *Penicillium expansum* with *P450-4* (Accession No. Q701P2.1) of *Gibberella fujikuroi* involved in the biosynthesis of gibberellins. b. Alignment of the

deduced amino acid sequence of *p450-2* (Accession No. DQ084390) of *Penicillium expansum* with *P450-4* (Accession No. Q701P2.1) of *Gibberella fujikuroi* involved in the biosynthesis of gibberellins.

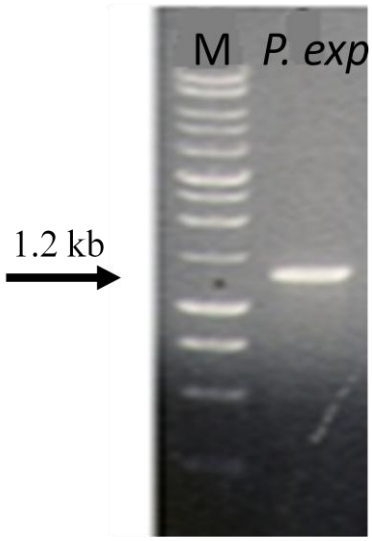


Figure 13: Amplification of the *gpe1* using the primers *mhsF* and *mhsR* in *P. expansum*. M: 1 kb DNA ladder.

3.1.4. Alignment of *gpe1* with other cytochrome P450 monooxygenases

In order to identify the other genes having resemblance with *gpe1* gene in the database, we performed a Blastx. As a result of this BLAST we identified seven cytochrome P450 monooxygenase: *Pax P* of *Penicillium paxilli*, *PbP450-2* of *Phoma betae*, *P450-4* of *Gibberella fujikuroi* and *P450-4* of *Sphaceloma manihoticola*, *ltm K* and *ltm J* of *Neotyphdium lolii* and *P450-1* of *Botryotinia fuckeliana* showing a high resemblance with *gpe1* of *P. expansum*. Then, we performed the alignment of the deduced amino acid sequence (394 residues) of *gpe1* with the seven identified cytochrome P450 monooxygenases. This alignment showed an average identity of 40 % of the amino acid sequence of gpe1 to the central and N-terminal parts of *PbP450-2* and *P450-4* enzymes

which have been found involved in indole diterpene synthesis and in gibberellin synthesis respectively and an average identity 37 % with other cytochromes P450 genes (Figure 14). So, these results lead to the formation of a hypothesis that the *gpe1* gene could be involved in the synthesis pathway of geosmin.

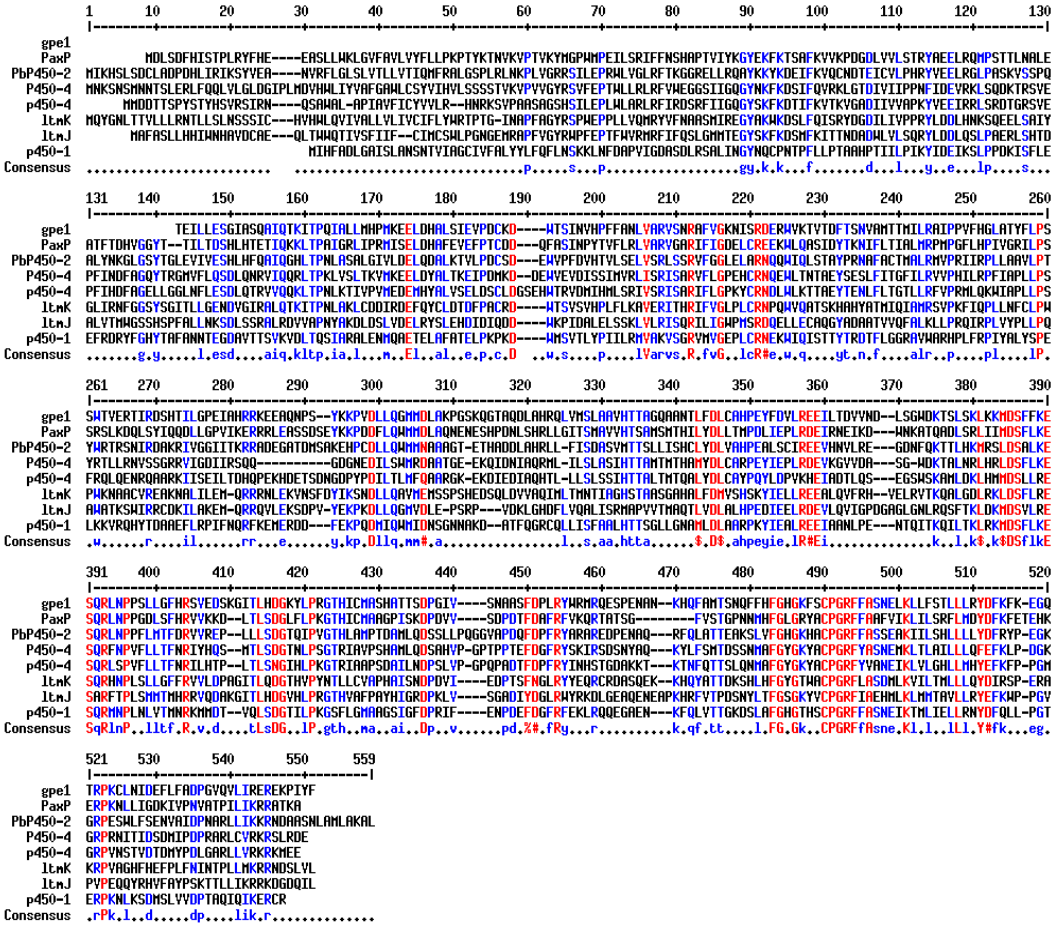


Figure 14: Alignment of the deduced amino acid sequence of *gpe1* with other cytochrome P450 monooxygenases genes: *Pax P* (Accession No. AAK11528) of *Penicillium paxilli* involved in the biosynthesis of paxilline, *PbP450-2* (Accession No. BAD29968) of *Phoma betae* involved in the biosynthesis of aphidicolin, *P450-4* (Accession No. Q701P2.1) of *Gibberella fujikuroi* and *P450-4* (Accession No. CAP07652.1) of *Sphaceloma manihoticola* involved in the biosynthesis of gibberellins, *ltm K* (Accession No. AAW88512) and *ltm J* (Accession No. ABF20221.1) of *Neotyphdium lolii* involved in the

biosynthesis of lolitrem and P450-1 (Accession No. CAP58781.1) of *Botryotinia fuckeliana*, was the direct target of Ga subunit BCG1.

3.1.5. *gpe1* gene presence in geosmin producing *Penicillium* species

To strengthen the hypothesis i.e. a possible role of gpe1 protein as a CYP involved in geosmin biosynthesis, we performed other PCRs on the fourteen *Penicillium* spp. using the same primers *mhsF* and *mhsR*. The results of these PCRs showed that the ten *Penicillium* spp. including *P. expansum* which were producers of geosmin gave the same ~1.2 kb fragment whereas, in the rest of *Penicillium* spp. which were non-producers of geosmin, either the band was not detected or it was less than 1.2 kb & very feeble (Figure 15). These results were quite intriguing to the involvement of *gpe1* in production of geosmin.

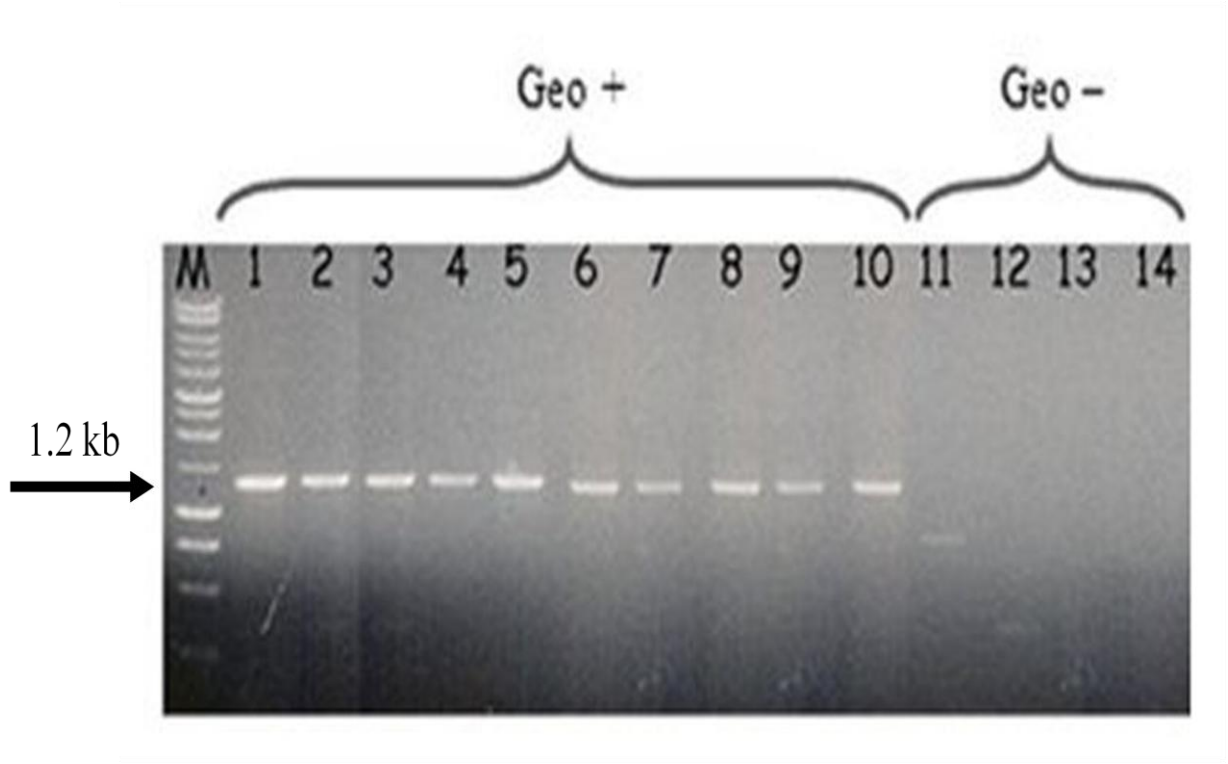


Figure 15: *gpe1* PCR amplification on geosmin productive (1-10) and non-productive (11-14) *Penicillium* species: 1. *P. aureo-cinnamomeum*, 2. *P. sclerotiorum*, 3. *P. spinulosum*, 4. *P. bilaiae*, 5. *P. echinalutum*, 6. *P. canescens*, 7. *P. paraherquei*, 8. *P.*

expansum, 9. *P. minioluteum*, 10. *P. geastrivorus*, 11. *P. brevicompactum*, 12. *P. ochrochoron*, 13. *P. restrictum*, 14. *P. crustosum*, M: 1 kb DNA ladder.

3.2. How to explore different aspects of the *gpe1* gene using bioinformatics tools?

3.2.1. Implication of P450 enzymes in the biosynthesis of different secondary metabolites

The biosynthetic pathways of fungi leading to the production of secondary metabolites are rather complex. These fungal biosynthesis pathways consist of several oxygenation steps catalyzed by different enzymes. Mycotoxins (i.e. aflatoxins, trichothecenes, and fumonisins) and higher plant hormones (i.e. gibberellins) are the best studied fungal secondary metabolite pathway. Five cytochrome P450 monooxygenases: CYP58, CYP59, CYP60, CYP62 and CYP64 are responsible for the biosynthesis of aflatoxin in *Aspergillus parasiticus*. These enzymes are mostly encoded in gene clusters and their expression is usually co-regulated. Four of these enzymes: CYP58, CYP59, CYP60 and CYP64 catalyze the oxidative reactions like epoxidation, oxidation, hydroxylation and desaturation respectively, whereas a reaction with a yet unknown mechanism is catalyzed by CYP62 (Bhatnagar *et al.*, 2003; Ehrlich *et al.*, 2004; Wen *et al.*, 2005).

Two P450 enzymes i.e. Fum2 and Fum6 are involved in the biosynthesis of fumonisins in *Fusarium verticillioides*. The enzyme Fum2 is responsible for the introduction of a hydroxyl group at carbon C-10 of the polyketide chain of these mycotoxins whereas, Fum6 catalyzes two consecutive hydroxylations at carbons C-14 and C-15 (Bojja *et al.*, 2004; Butchko *et al.*, 2006). Four P450 enzymes i.e. P450-1, P450-2, P450-3 and P450-4 are involved in the biosynthesis of gibberellins in *Fusarium fujikuroi*. These P450 enzymes are

multifunctional and catalyze 10 of the 15 reaction steps (Rojas *et al.*, 2001; Tudzynski *et al.*, 2001; 2002).

In *Streptomyces coelicolor* A3, the *sco5223* gene encodes a unique bifunctional P450 i.e. CYP170A1. This enzyme can catalyze two sequential oxidation reactions of the terpenoid epi-isozizaene through two epimers of albaflavenol to the single keto sesquiterpene product, albaflavenone. Interestingly, a terpenenoid synthase activity to generate farnesene isomers from farnesyl diphosphate is also shown by CYP170A1. It is less clear how and when the organism might trigger farnesene synthesis. Therefore, CYP170A1 is clearly a moonlighting protein (Zhao and Waterman 2011).

3.2.2. Conserved domains of cytochrome P450s

In P450s, the highest structural conservation is found in the core of the protein around the heme. A four-helix (D, E, I and L) bundle, helices J and K, two sets of β sheets, and a coil called the meander form the conserved core. These regions comprise the heme-binding loop, Glu-X-X-X-Arg motif and the central part of the I helix, containing a consensus sequence considered as P450 signature (Ala/Gly-Gly-X-Asp/Glu-Thr-Thr/Ser), which corresponds to the proton transfer groove on the distal side of the heme. Most eukaryotic P450s are associated with microsomal membranes, and very frequently have a cluster of prolines (Pro-Pro-X-Pro) that form a hinge, preceded by a cluster of basic residues between the hydrophobic amino-terminal membrane anchoring segment and the globular part of the protein (Figure 16). The most variable regions are associated with either amino-terminal anchoring or targeting of membrane-bound proteins, or substrate binding and recognition (Werck-Reichhart and Feyereisen, 2000).

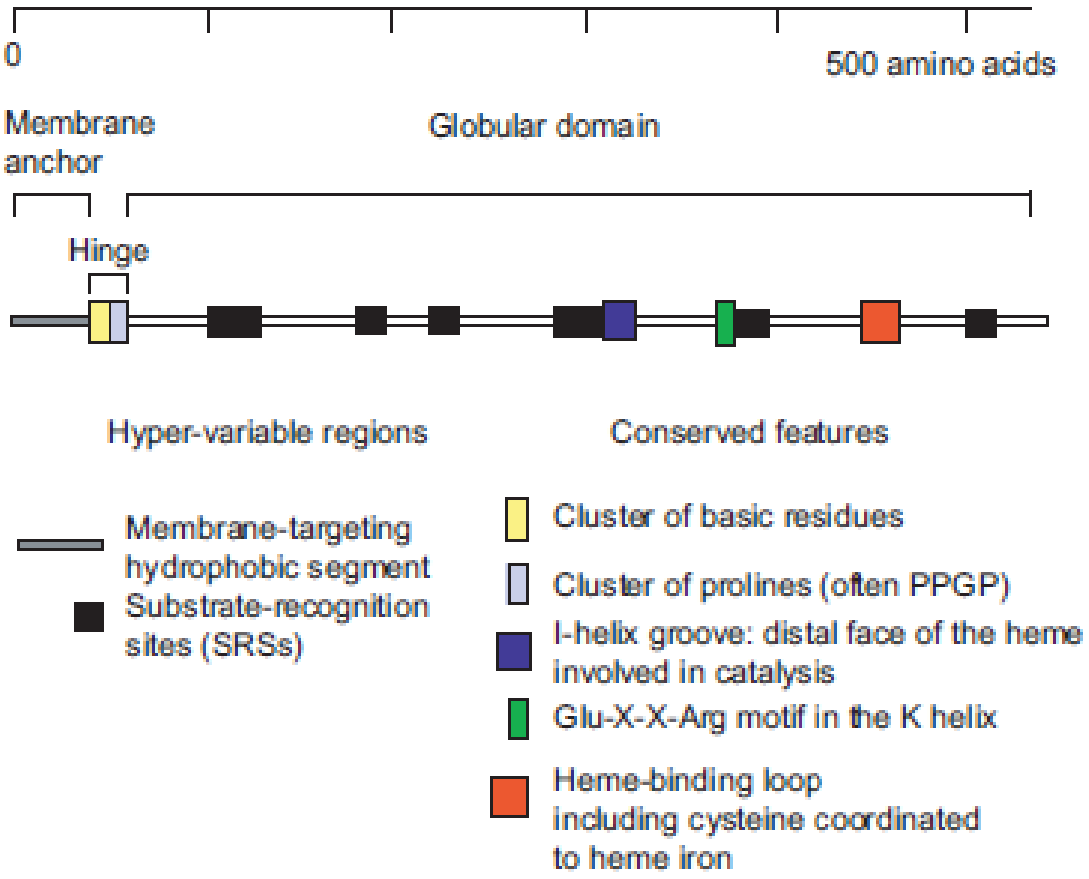


Figure 16: Typical features of an ER-bound P450 protein.

3.2.3. Which functional domains of cytochrome P450 monooygenase enzymes are present in *gpe1*?

The *gpe1* gene was analyzed to know about its structural conservation. Among the absolutely conserved domains of cytochromes P450 monooxygenases (CYPs), the heme-binding loop (327-336 aa), the Glu-X-X-Arg motif (240-243 aa) and the Trp-X-X-X-Arg (116-120 aa) are also found present in the corresponding amino acid sequence (394 residues) of *gpe1* (Figure 17). The heme-binding loop, containing the most characteristic P450 consensus sequence, located on the proximal face of the heme just, with the absolutely conserved cysteine that serves as fifth ligand to the heme iron. The conserved Glu-X-X-Arg motif in helix K, also on the proximal side of heme is probably needed to stabilize the core structure (Werck-Reichhart and Feyereisen, 2000).

1 TEILLESGIA SQAIQTKITP QIALLMHPMK EELDHALSIE VPDCKDWTSI NVHPFFANLV

61 ARVSNRAFVG KNISRDERWV KTVTDFTSNV AMTTMILRAI PPVFHGLATY FLPSSWTVER

121 TIRDSHTILG PEIAHRRKEE AQNPSYKKPV DLLQGMMDLA KPGSKQGTAQ DLAHRQLVMS

181 LAAVHTTAGQ AANTLFDLCA HPEYFDVLRE EILTDVVNDL SGWDKTSLSK LKKMDSFFKE

241 SQRLNPPSLL GFHRSVEDSK GITLHDGKYL PRGTHICMAS HATTSDPGIV SNAASFDPLR

301 YWRMRQESPE NANKHQFAMT SNQFFHFGHG KFSCPGRFFA SNELKLLFST LLLRYDFKFK

361 EGQTRPKCLN IDEFLFADPG VQVLIREREK PIYF

Figure 17: Conserved domains of cytorochromes P450 monooxygenases present in *gpe1*. Trp-X-X-X-Arg motif shown in blue color and underlined, Glu-X-X-Arg motif shown in green color and underlined and heme binding loop shown in red color and under lined. W (Trp): tryptophan, R (Arg): arganin, (E) Glu: glutamic acid.

Then, we searched different motif databases using different sites, in order to find the motifs of *gpe1* sequence. The InterPro Search (European Bioinformatics Institute, United Kingdom) using FPrintScan matched the gpe1 protein with a cytochrome P450 of class E and group IV which showed four conserved motifs (aa 231-247, aa 284-302, aa 318-334 and aa 334-352) having activities as monooxygenase activity, iron ion binding, heme binding and oxidation-reduction process (Figure 18).

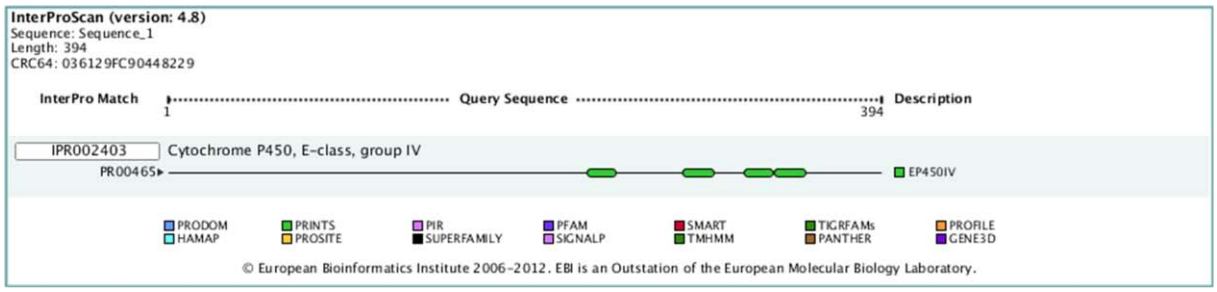


Figure 18: InterPro Scan visual output showing different domain of *gep1*.

3.3. Does the *gpe1* gene require for the geosmin biosynthesis in *P. expansum*

3.3.1. Production of mutants by the gene disruption method and their screening by PCRs

To verify the involvement of *gpe1* gene in the biosynthesis of the geosmin, the *gpe1* was functionally characterized in *P. expansum* M 2230. The first step of this characterization was the construction of mutants of the *gpe1* gene by gene disruption method. In order to produce mutants of *gpe1* gene, *P. expansum* M 2230 protoplasts were transformed with TopoPhph vector (the vector containing the *gpe1* gene disrupted by the integration of *hph* cassette in it) (Figure 9b). Forty two transformants which were able to grow on YES medium supplemented with hygromycin were subsequently screened by three consecutive PCRs to monitor integration of *hph* cassette in the genome of *P. expansum*.

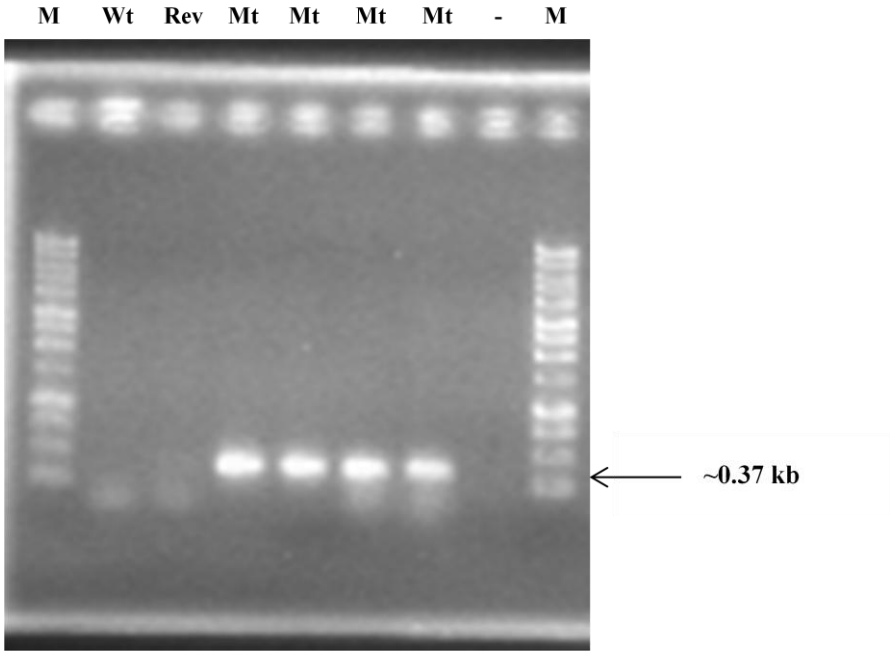
A PCR using primer pair *hphF/hphR* on positive tranformants resulted into a fragment of ~0.37 kb corresponding to *hph* cassette in only four transformants (Mt) (Figure 19a). These four transformants (Mt) were subjected to the second PCR using primers *mhsF* and *hphR* that gave a ~1.5 kb *gpe1/hph* shared fragment (Figure 19b) and then, a third PCR on the same four transformants (Mt) using primers *hphF* and *mhsR* resulted into the amplification of a ~1.1 kb *hph/gpe1* shared fragment (Figure 19b). No amplification was

observed in the wild type *P. expansum* (Wt) with any of the primers combination (Figure 19a and 19b).

3.3.2. Production of reverse complements and their screening by PCRs

To obtain reverse complements of *gpe1* gene, protoplasts of *Δgpe1* mutant were transformed with TopoP vector (the vector containing the *gpe1* gene) (Figure 9c). The transformants which grew only on YES medium but not on YES medium supplemented with hygromycin were selected. These selected transformants were subjected to the same three screening PCRs using primer pairs *hphF/hphR*, *mhsF/hphR* and *hphF /mhsR*. No amplification product in complementary mutants (Rev) with any of the primer pairs depicted the removal of the *hph* cassette from the genome of *P. expansum* (Figure 19a and 19b).

a.



b.

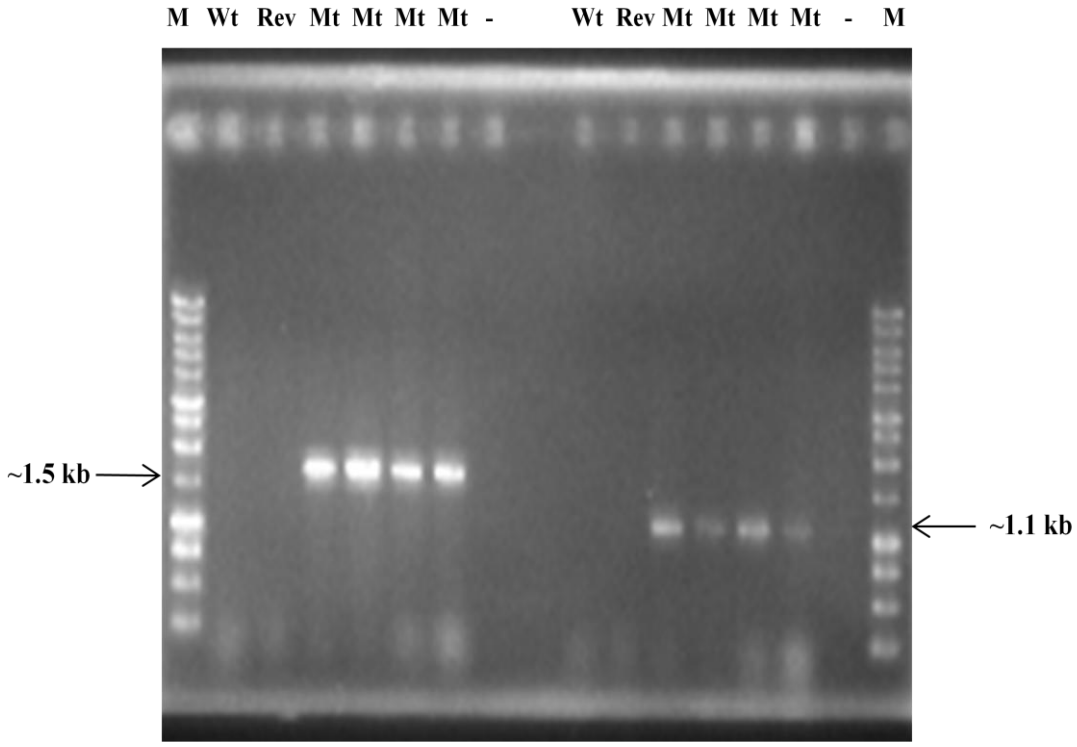


Figure 19: PCR transformants screening. a. *P. expansum* wild type (Wt), *gpe1* complementary mutant (Rev) and *Δgpe1* mutants (Mt) with primers *hphF/hphR*. b. *P. expansum* wild type (Wt), *gpe1* complementary mutant (Rev) and *Δgpe1* mutants (Mt) with primers *mhsF/hphR* and *hphF/mhsR*. **-** : negative control. M: 1kb DNA ladder.

3.3.3. Quantification of geosmin by gas chromatography–mass spectrometry (GC/MS)

By the construction of *Δgpe1* mutant, the *gpe1* gene remained no more functional in the *P. expansum* 2230. Therefore, to know about its role in the production of geosmin in *P. expansum*, we launched a culture of *Δgpe1* mutant to see the production of geosmin. To quantify the production of geosmin, the samples were sent to Exact Laboratory at Macon, France where they were analyzed by gas chromatography–mass spectrometry (GC/MS). The results of the geosmin quantification analysis showed that *Δgpe1* mutant lost its power to produce geosmin (Table 4). The lost of geosmin production by *Δgpe1* mutant described the

involvement of *gpe1* gene in the geosmin biosynthesis pathway. Then, the *gpe1* gene was made functional by producing its reverse complements. The same geosmin quantification analysis was performed for the reverse complement of the *gpe1* gene. The geosmin was produced in the reverse complement of *gpe1* like that of *P. expansum* wild type (Table 4) which means the reverse complement regained its power to produce geosmin which was lost in *Δgpe1* mutant. So, in light of above results, the involvement of *gpe1* gene in the biosynthetic pathway of geosmin is evident.

Table 4: Dosage of geosmin.

Compound	Geosmin
Unit	(ng/L)
LQ	10
Wild type	14
Reverse complement	14
Mutant	<10 ng / L

LQ = Limit of quantification

3.4. Where does the *gpe1* gene intervene in the biosynthesis pathway of geosmin?

3.4.1. Dependence of secondary metabolites pathways upon the relative abundance of their precursors

The terpenes produced in *Streptomyces* species seem to be derived from either the mevalonate-dependent or mevalonate-independent pathways (Rhomer, 2003; Takahashi *et al.*, 1998). The simple C5-unit isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP) are the initial substrates for terpene biosynthesis. These five-carbon isoprene units are derived from pyruvate or acetyl-CoA (Figure 20) which in turn are the products of central carbon metabolism. The acetyl-CoAs are the precursors for the

production of many secondary metabolites. The biosynthetic pathways for secondary metabolism, therefore, depend upon the relative abundance of these precursors. Blocking the consumption of precursors in one pathway may induce another biosynthetic pathway.

Singh *et al.* (2009) observed an elevated production of the intracellular pool of acetyl-CoA after deletion of the doxorubicin biosynthetic pathway led to enhanced growth and longer survival of cell culture. Likewise, the greater accumulation of acetyl-CoA led to the biosynthesis of geosmin in *Streptomyces peucetius*. As the concentration of geosmin synthase was increased, production of geosmin increased concurrently in the presence of sufficient acetyl-CoA. The rate of enzyme activity increases in direct proportion to the increase in substrate concentration.

The increase in enzyme concentration increases the products simultaneously. The nysF inactivation that increases nystatin production in *S. noursei* is another case of deletion of biosynthetic genes leading to raise secondary metabolite production (Volokhan *et al.*, 2005). So, keeping in view the production of geosmin depending on the availability of acetyl CoA, with an interaction with doxorubicin synthesis (Sing *et al.*, 2009), 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.

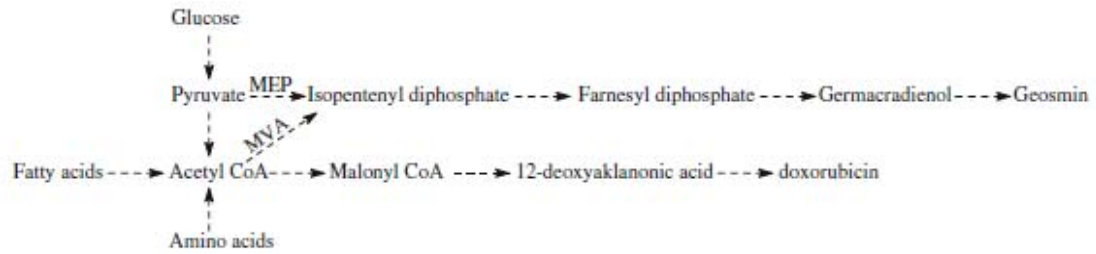


Figure 20: Metabolic pathway diagram the mevalonate (MVA) and non-mevalonate (MEP) pathways that link geosmin synthesis.

3.4.2. Is the biosynthesis pathway of geosmin same in *P. expansum* as that suggested in bacteria?

In bacteria, the geosmin biosynthetic pathway is well explained in which a single bifunctional enzyme i.e. germacradienol/geosmin synthase catalyzes the conversion of farnesyl diphosphate (FPP) which is the precursor of sesquiterpenes, into geosmin in a two step reaction (Cane *et al.*, 2006; Ghimire *et al.*, 2008; Jiang *et al.*, 2006; Giglio *et al.*, 2008). On the contrary, the biosynthesis pathway of geosmin has not yet been characterized in eukaryotes till today. Therefore, the results of the BLAST search with the protein sequence of the *S. coelicolor A3* (SCO 6063) which encodes the germacradienol/geosmin synthase as a query showing no genes in the genus *Penicillium* having homology with the genes encoding germacradienol/geosmin synthase are quiet sufficient to propose that the biosynthesis of geosmin may follow a different pathway in *P. expansum* in place of that one followed in bacteria.

3.4.3. Use of *Penicillium marneffei* genome to know about the neighbor genes of the *gpe1*

In order to know that what type of genes are present in the neighborhood of *gpe1* gene, we used the genome of *Penicillium marneffei* whose genome has been completely sequenced. We took the nucleotide sequence of *gpe1* and performed a blastx in *Penicillium marneffei* (taxid:37727). This blast showed the highest value of identity with PMAA_088100 a gene (consisting of 1611 bp) encoding a putative cytochrome P450 monooxygenase of *P. marneffei* ATCC 18224 (Figure 21). Then, we took the nucleotide sequence of the gene on the right side of the PMAA_088100 and performed a tblastx. In this way, we searched 5 genes on each side of the PMAA_088100 (Figurers 21). The purpose of this search was to see the some neighboring genes of the *gpe1* which could help us in better

understanding of geosmin pathway in *P. expansum*. On the right side of the cytochrome P450 monooxygenase (PMAA_088100), two interesting genes encoding a putative polyketide synthase (PMAA_088150) and a putative NRPS-like enzyme were found.

Polyketide synthases (PKS) are large multi-enzyme protein complexes that contain one and/or many of the following functional domains: ketosynthase (KS), acyl transferase (AT), ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), methyltransferase (MT), acyle carrier protein (ACP) and thioesterase (TE). However, the three principle domains are AT, KS and ACP while the remaing domains are optional (Fujii *et al.*, 1998). The PKS act in a step-wise manner to biosynthesize the corresponding polyketides, such as pigments and mycotoxins, from simple 2-, 3-, 4-carbon building blocks such as acetyl-CoA, propionyl CoA, butyryl-CoA and their activated derivatives, malonyl-, methylmalonyl- and ethylmalonyl-CoA (Khosla *et al.*, 1999).

Nonribosomal peptide synthetases (NRPS) are multimodular enzymes that make nonribosomal peptides through a thiotemplate mechanism independent of ribosomes. A unique feature of NRPS system is the ability to synthesize peptides containing proteinogenic as well as non-proteinogenic amino acids. In many cases these enzymes work in conjunction with polyketides synthases giving hybrid products. The products of these multifunctional enzymes have a broad spectrum of biological activities, some of which have been useful in medicine, agriculture, and biological research (Kleinkauf and Doehren, 1996; Schwarzer and Marahiel, 2001; Smith *et al.*, 1990).

All of three i.e. the cytochrome P450 monooxygenase, PKS and NRPS are the enzymes involved in the biosynthesis of different secondary metabolites. So, on the basis of this search in silico, we can say that *gpe1* gene might be the part of a gene cluster encoding the enzymes involved in the biosynthesis of secondary metabolites.

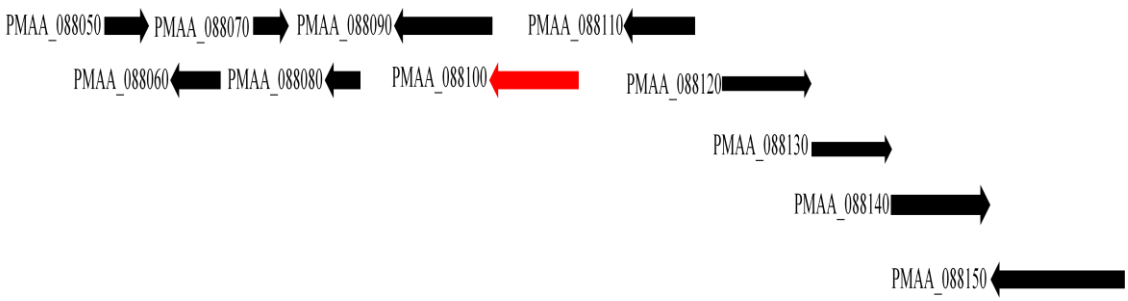


Figure 21: A simple diagram showing neighbor genes of the particular gene of *Penicillium marneffei* showing highest resemblance with *gpe1*of *P. expansum* as a result of BLAST search. PMAA_088100: a 1611 bp gene encoding a putative cytochrome P450 monooxygenase. PMAA_088110: a 1035 bp gene encoding a hypothetical protein. PMAA_088120: a 1689 bp gene encoding a putative NRPS-like enzyme. PMAA_088130: a 1295 bp gene encoding a metallo-beta-lactamase superfamily protein. PMAA_088140: a 2084 bp gene encoding an abc1 domain protein. PMAA_088150: a 11961 bp gene encoding a putative polyketide synthase. PMAA_088090 (2476 bp), PMAA_088080 (786 bp), PMAA_088070 (600 bp) and PMAA_088060 (1014 bp), all encode a hypothetical protein whereas PMAA_088050 (888 bp) encodes a putative NAD dependent epimerase/dehydratase.

Chapter IV

General Conclusions and Future Prospects

4.1. General Conclusions

Geosmin is a powerful aromatic compound having an earthy smell and is associated with off-flavors in water and wine (Gerber and Lechevalier, 1965; Darriet *et al.*, 2000). In case of water, contamination is strictly caused by bacteria (Jüttner and Watson, 2007). In case of wine, the development of *Penicillium expansum* on grapes, with a possible interaction of *Botrytis cinerea* is the principal cause of geosmin origin (La Guerche *et al.*, 2004; Morales-Valle *et al.*, 2011). Its presence is highly detrimental to the aromatic quality of wine due to its low olfactory perception threshold and stability during aging (Darriet *et al*., 2000; 2001). Different removal or degradation processes of geosmin cannot be applied in wines as they would be detrimental to their organoleptic quality.

In this context, a better knowledge of the geosmin biosynthesis pathway in filamentous fungi *P. expansum* will help to define new strategies to reduce contamination in grapevine products. The geosmin biosynthesis pathway has been well characterized in bacteria particularly in the genus *Streptomyces*. In bacteria, a bifunctional enzyme germacradienol/geosmin synthase catalyzes the Mg^{2+}-dependent conversion of farnesyl diphosphate (precursor of cyclic sesquiterpenes) into germacradienol and germacrene D and geosmin (Jiang *et al.*, 2007). According to our knowledge no gene encoding germacradienol/geosmin synthase has been characterized in the eukaryotes until today. We performed a bioinformatics search in order to determine the presence of germacradienol/geosmin synthase in the databases of the genus *Penicillium*, which did not show any gene having homology with the genes encoding germacradienol/geosmin synthase. So, it is evident that there is no gene encoding germacradienol/geosmin synthase, present in the genus *Penicillium*. Therefore, some other enzymes might be involved in geosmin production. These enzymes could also be cytochrome P450 monoxygenases as one cytochrome P450 (CYP180A1) has already been predicted to be involved in the biosynthesis

of geosmin (Lamb *et al.*, 2003). Moreover, cytochrome P450s have also been found in terpene synthesis (Kimura et al., 2007; Agger et al., 2008; Zhao et al., 2009). In the contrary, White *et al.* (2006) proposed that two cytochrome P450 gene fragments i.e. *p450-1* and *p450-2* involved in patulin biosynthesis in *P. expansum*. We performed an alignment of the deduced amino acid sequences of the cytochrome P450 gene fragments i.e. *p450-1* and *p450-2* of *P. expansum* with the CYP619C2 and CYP619C3 (cytochrome P450s) involved in patulin biosynthesis in *Aspergillus clavatus* (Artigot *et al.*, 2009), which showed a very weak identity (Figure 10) among the P450 genes of *P. expansum* and the P450 genes of *Aspergillus clavatus* needed for patulin synthesis. As we were wondering about the involvement of *p450-1* and *p450-2* in the biosynthesis of geosmin, therefore, we performed the alignments of *p450-1* and *p450-2* gene fragments of *P. expansum* with a gene involved in terpene biosynthesis which showed a high identity (about 40 %) of *p450-1* and *p450-2* with the terpene synthase (Figure 11a, b). So, in the light of the results of these alignments, we can say that *p450-1* and *p450-2* may have another role in *P. expansum* rather than the involvement in patulin synthesis. The result of the PCR performed on *p450-1* and *p450-2* illustrated that these DNA fragment belongs to a single cytochrome *p450* gene of 1182 bp (Figure 12) that we named as *gpe1*.

An alignment of the deduced amino acid sequence (394 residues) of the *gpe1* with the seven identified cytochrome P450 monooxygenases displayed an average identity of 40 % of the amino acid sequence of *gpe1* with PbP450-2 and P450-4 enzymes which have been found involved in indole diterpene synthesis and in gibberellins synthesis respectively, and an average identity of 37 % with other cytochromes P450 genes (Figure 13). Then, the results of PCRs performed on the fourteen *Penicillium* spp. using the same primers which were used for *gpe1* amplification showed that only *Penicillium* spp. which were producers of geosmin gave the same ~1.2 kb fragment (Figure 14) like *gpe1* and the nucleotide sequences of these DNA

fragments had > 98 % similarity with *gpe1* of *P. expansum*. So, from above results, we can infer that the *gpe1* gene could be involved in the geosmin synthesis pathway.

We performed an analysis of the *gpe1* gene in order to explore its different aspects which enabled us to identify the presence of some conserved domains of cytochrome P450s in gpe1 sequence (Werck-Reichhart and Feyereisen, 2000). Then, we performed the functional characterization of the *gpe1* gene in *P. expansum* M2230. The loss of the potential to produce geosmin in *Δgpe1* mutant and then, regain the potential to produce geosmin by the reverse complement of *gpe1* was sufficient to conclude the involvement of the *gpe1* gene encoding a cytochrome P450 monooxygenase in the biosynthesis of geosmin. In order to know that which type of genes are present in the neighborhood of *gpe1* gene, we performed a search with nucleotide sequence of *gpe1* in the genome of *Penicillium marneffei* using bioinformatics tools. The presence of two interesting genes encoding a putative polyketide synthase and a putative NRPS-like enzyme on the right side of the cytochrome P450 monooxygenase suggested to say that *gpe1* gene might be the part of a gene cluster encoding the biosynthesis of secondary metabolites as all of three i.e. the cytochrome P450 monooxygenase, PKS and NRPS are the enzymes involved in the biosynthesis of different secondary metabolites.

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

4.2. Future Prospects

We have performed the functional characterization of a gene involved in the biosynthesis of the geosmin in *P. expansum*. As prospectives of this thesis, different points can be considered.

1. The characterization of the other genes of the biosynthesis pathway of the geosmin in the *P. expansum* would be done.
2. In this context, the gene bank of *P. expansum* would be constructed.
3. Then, screening of this gene bank of *P. expansum* would be performed using different radioactive probes including *gpe1*.
4. If some interesting genes will be found in the cluster encoding the biosynthesis of the geosmin as a result of screening of the gene bank of the *P. expansum*, then, the expression of those genes would be studied by producing their mutants and reverse complements.
5. The verification of the sequence and distribution of *gpe1* gene in different isolates of *P. expansum* producers and non-producers of geosmin would be performed.
6. It would be interesting to develop a rapid test for the characterization of *P. expansum* in order to characterize this species directly on grapes by using a single molecular test.
7. It would also be interesting to use the Q-PCR for the indicrect quantification of geosmin from the grape samples would be performed.

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Annexes

Annexe 1. Publication accepted

Annexe 1. Publication accepted

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Characterization of a cytochrome p450 monooxygenase gene involved in the biosynthesis of geosmin in *Penicillium expansum*

Muhammad Hussnain Siddique[1,2], Thierry Liboz[1,2], Nafees Bacha[3], Olivier Puel[4], Florence Mathieu[1,2], Ahmed Lebrihi[1,2,5*]

[1] Université de Toulouse, INPT-UPS, Laboratoire de Génie Chimique, avenue de l'Agrobiopole, 31326 Castanet-Tolosan Cedex, France.

[2] Le Centre national de la recherche scientific (CNRS), Laboratoire de Génie Chimique, 31030 Toulouse, France.

[3] Centre of Biotechnology and Microbiology, University of Peshawar, Pakistan.

[4] Institut National de la Recherche Agronomique (INRA), Laboratoire de Pharmacologie Toxicologie, 31931 Toulouse, France.

[5] Université Moulay Ismail, Marjane 2, BP 298, Meknes, Maroc.

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Abstract

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), In this case, this molecule can be removed by the physical, chemical and biological treatments (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 cannot be applied. Nowadays, predictive models of fungal growth are therefore the best way to control geosmin production (Judet-Correia *et al.*, 2010).

In this context, a better knowledge of the geosmin biosynthesis pathway in filamentous fungi will help to define new strategies to reduce contamination in grapevine products. This pathway is well characterized in bacteria, especially in the genus *Streptomyces*. A bifunctional germacradienol/geosmin synthase catalyze the conversion of farnesyldiphosphate, a primary metabolite, into geosmin in a two-step process (Jiang *et al.*, 2007). Until today, no germacradienol/geosmin synthase has been characterized in fungi species to our knowledge. Bioinformatics analysis (screening of genes encoding this enzyme) gave no results in the genus *Penicillium*, suggesting that this enzyme, if present in *P. expansum*, is different from those described in bacteria. Geosmin structure, and the presence of one hydroxyl group (Figure 1), may lead to other enzymes, like cytochrome P450 monooxygenases, as has been suggested in bacteria (Lamb *et al.*, 2003). These enzymes are involved in many metabolic pathways, including the biosynthesis of terpenes and their derivatives (Cresnar and Petric, 2011).

During 2006 White *et al.* (2006) characterize two DNA fragments, *p450-1* and *p450-2*, corresponding to parts of putative cytochrome P450 monooxygenase genes in *P. expansum* (strain IBT 21771) by suppression subtractive hybridization. As these two fragments were isolated from population of transcripts preferentially expressed under patulin-permissive conditions, the authors concluded to their involvement in patulin biosynthesis.

More recently, the two cytochrome P450 genes needed for patulin biosynthesis were functionally characterized in *Aspergillus clavatus* (Artigot *et al.*, 2009). Sequences

alignments revealed weak identities (28%) between these 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

Penicillium 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, grounded 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 extraction of DNA from gel was performed by the QIAquick Gel Extraction Kit (QIAGEN, 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) i.e OD 260 nm / OD 280 nm and OD 260 nm respectively and by agarose (Promega, France) gel electrophoresis.

PCR amplifications

PCR amplifications were performed in 25 µL reaction mixtures containing 2.5 µL of *Taq* polymerase 10 X buffer with $MgCl_2$, 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 H_2O upto 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 T_m 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 pID2.1, 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 TopoP*hph* (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.

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 hours 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, Δ*gpe1* 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 hour. 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 discussions

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), to test the hypothesis that the two previously cloned DNA fragments belong to the same gene. 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) 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 farnesyldiphosphate (sesquiterpene) can probably lead to the formation of geosmin in a similar process. Farnesyldiphosphate 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, we first used the same primers *mhsF* and *mhsR* 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*, by the gene disruption method. To obtain mutants of *gpe1*, protoplasts issued from *P. expansum* M2230 cells were transformed with TopoP*hph* 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 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 *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 farnesyldiphosphate, 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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Table 1: PCR primers used in this study

Primer name	Sequence (5'–3')
mhsF	CGAAATTCTGCTGGAAAGCG
mhsR	ATTGGCTTTTCCCGTTCACG
hphF	GAATTCAGCGAGAGCCTGAC
hphR	ACATTGTTGGAGCCGAAATC

Figure legends

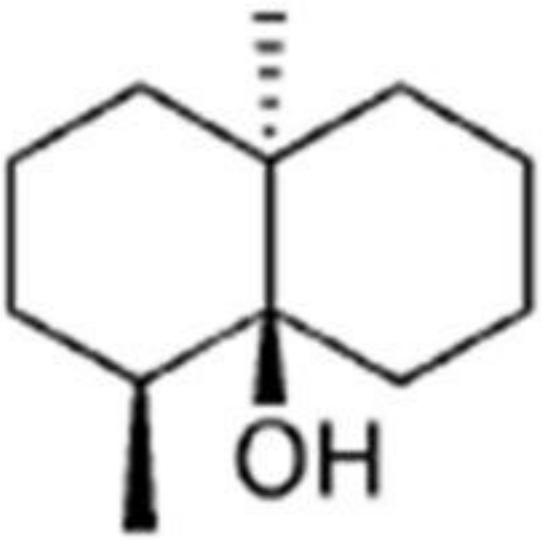
Figure 1. Chemical structure of geosmin

Figure 2. Schematic representation of transformation vector formation and *gpeI* gene disruption. (a) Using primer pair *mhsF*/*mhsR* (Table 1), 1182 bp *gpe1* gene containing *Sma*I 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 *Pml*I (indicated by triangle) to obtain *hph* cassette (1032 bp). TopoP was restricted with *Sma*1 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.

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, *ltm K* (Accession No. AAW88512) of *Neotyphdium lolii* involved in the biosynthesis of lolitrem, *PbP450-2* (Accession No. BAD29968) of *Phoma betae* involved in the biosynthesis of aphidicolin and *P450-4* (Accession No. Q701P2.1) of *Gibberella fujikuroi* involved in the biosynthesis of gibberellin.

Figure 4. *gpe1* PCR amplification on geosmin productive (1-10) and non-productive (11-14) *Penicillium* species: 1. *P. aureo-cinnamomeum*, 2. *P. sclerotiorum*, 3. *P. spinulosum*, 4. *P. bilaiae*, 5. *P. spinulosum*, 6. *P. canescens*, 7. *P. paraherquei*, 8. *P. expansum*, 9. *P. minioluteum*, 10. *P. geastrivorus*, 11. *P. brevicompactum*, 12. *P. ochrochoron*, 13. *P. restrictum,* 14. *P. crustosum,* M: 1 kb DNA ladder.

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: 1kb DNA ladder.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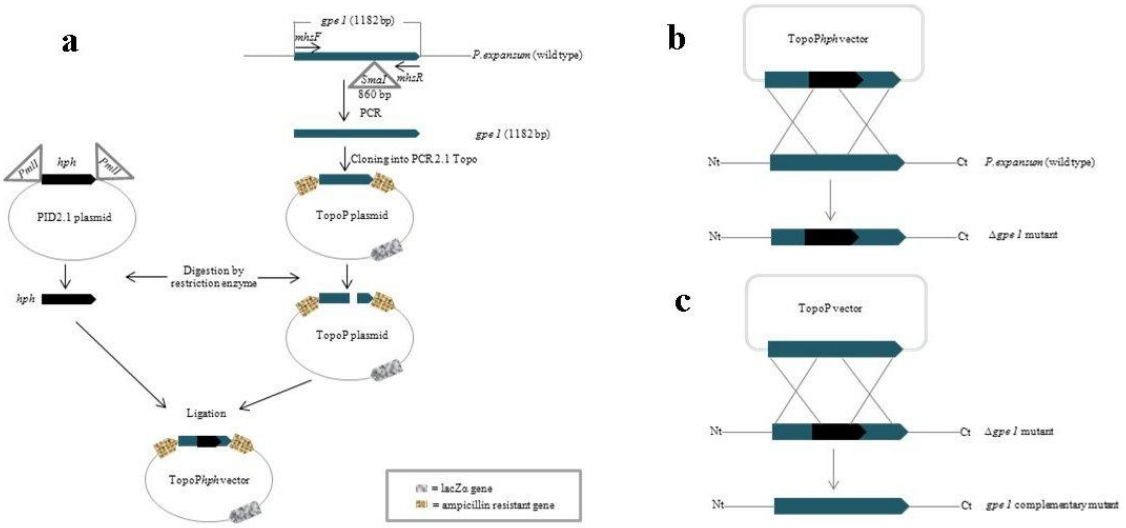
a
gpe1 (1182 bp)
mhzF
P. expansum (wild type)
SmaI
mhzR
860 bp
PCR
gpe1 (1182 bp)
Cloning into PCR 2.1 Topo
PmlI
hph
PmlI
PID2.1 plasmid
TopoP plasmid
Digestion by restriction enzyme
hph
TopoP plasmid
Ligation
TopoPhph vector
= lacZα gene
= ampicillin resistant gene
b
TopoPhph vector
Nt
Ct
P. expansum (wild type)
Nt
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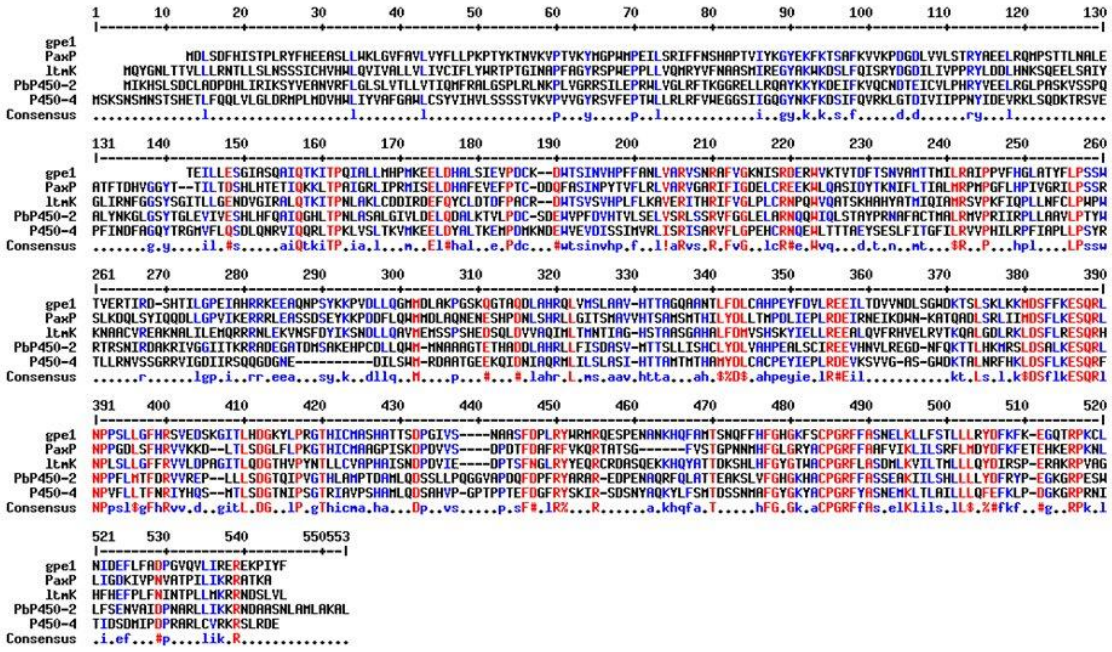

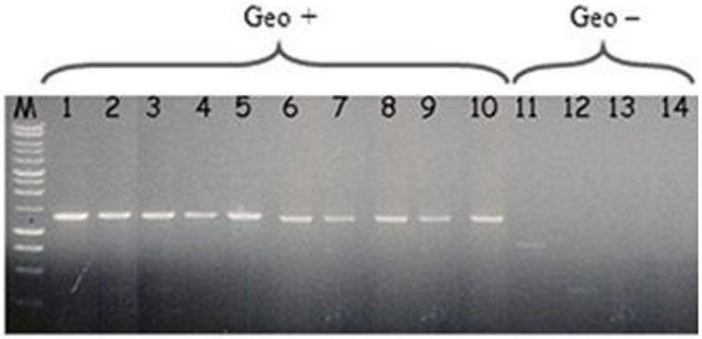
Geo +
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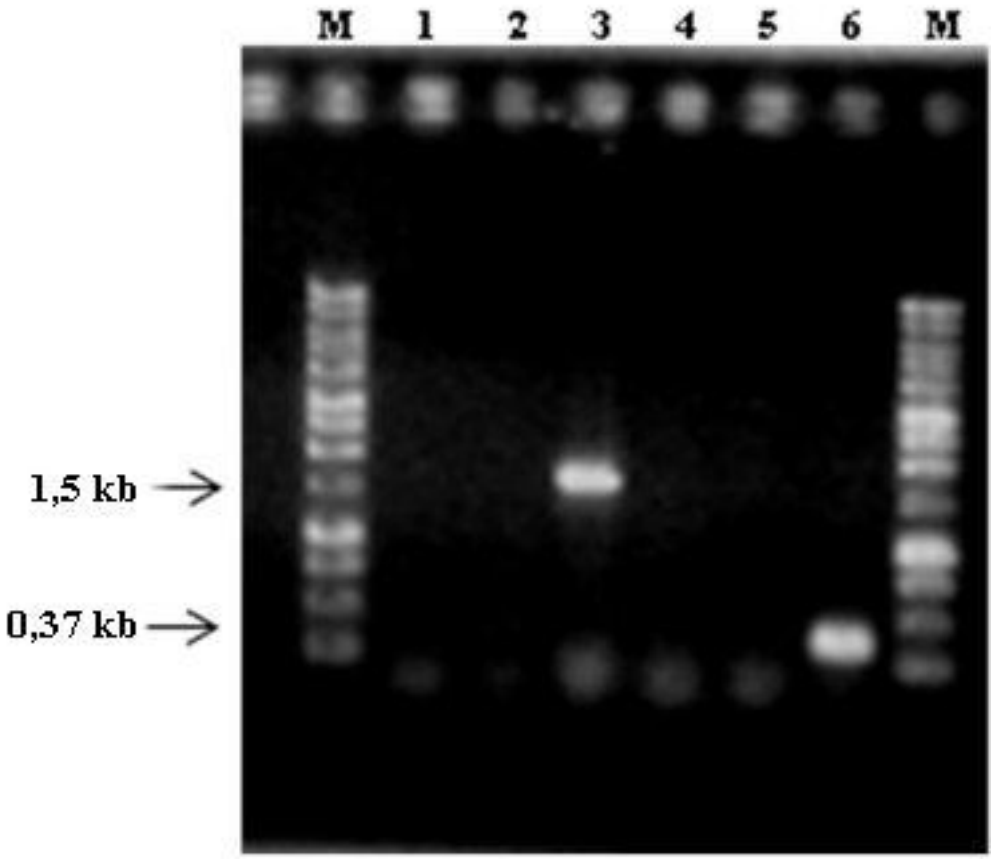
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14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