THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par *Institut National Polytechnique De Toulouse*
Discipline ou spécialité : *Microbiologie & Biocatalyse Industrielles*

Présentée et soutenue par *BACHA NAFEES*
Le *15/09/2009*

Titre : *CARACTERISATION DES POLYCETONES SYNTHASES INTERVENANT DANS LA BIOSYNTHESE D'OCHRATOXINE A, D'ACIDE PENICILLIQUE, D'ASPERLACTONE ET D'ISOASPERLACTONE CHEZ ASPERGILLUS WESTERDIJKIAE*

JURY

MATHIEU Florence	Professeur, Université de Toulouse	Président
FORGET Florence	Directeur de recherche,INRA, Bordeaux	Rapporteur
RATOMAHENINA Robert	Ingénieur de recherche,INRA, Montpellier	Rapporteur
BARREAU Christian	Chargé de Recherche CNRS, Bordeaux	Examinateur
PUEL Olivier	Ingénieur de recherche,INRA, Toulouse	Examinateur
LEBRIHI Ahmed	Professeur, Université de Toulouse	Directeur de these

---

Ecole doctorale : *École doctorale: Sciences Ecologiques, Vétérinaires, Agronomiques et Bioingénieries*
Unité de recherche : *Laboratoire de Génie Chimique*
Directeur de Thèse : *M. LEBRIHI Ahmed (Pr. ENSAT-INPT)*
Co-Directeur de Thèse : *Mme MATHIEU Florence (Pr. ENSAT-INPT)*
Rapporteurs : *Florence Richard-Forget et Robert Ratomahenina*

Acknowledgements

First of all I am thankful to the almighty ALLAH, whose blessing and guidance let me windup my thesis with good health. Second, my sincere appreciation goes to my supervisor Professor Ahmed LEBRIHI and co-supervisor Professor Florence MATHIEU, whose scientific approach, careful reading and constructive comments were valuable. Their timely and efficient contributions helped me shape my research work into its final form and I express my sincerest appreciation for their assistance in any way that I may have asked.

I also wish to thank the "Higher Education Commission (HEC)" of Pakistan, its leadership and the staff who in their limited resources supported me financially for my studies in France. I must also acknowledge the way HEC remained in contact with me and addressed what so ever problem I have faced during my stay. The placement of new doctorate degree holders was not an easy task. I appreciate the efforts of HEC to provide me an opportunity to serve my country. I must also mention the services provided by SFERE (Société française d'exportation des ressources éducatives) to facilitate my living in France.

I am also indebted to my colleagues at "Laboratoire de Genie Chemique (LGC)", particularly Patricia NOUVET, Thierry LIBOZ, Morie Cormen MONJE, Alaoui Lamrani HAFSA, Rafik ERRAKHI and Samia AFOULOUSE who always welcomed, helped and appreciated me. I am also thankful to Ali ATOUI, Anne-Claire Chorin Gwenaelle JARD, Muhammad SHAHID and Muhammad ARSHAD for their guidance and support. I am grateful to all my Pakistani friends specially "Muhammad Ali Nizamani" for reminding me that I am not away from my family. Special thanks, tribute and appreciation to all those their names do not appear here who have contributed to the successful completion of this study.

Finally, I'm forever indebted to my family, who prioritized my studies over all family related duties. My humble thanks to my mother, late father, wife Asma, brother Hafiz-ul-Asad, Cousin Syed Tamiz-ud-din, sisters Afsheena, Neelam, Nighat and Farkhanda and last but not the least my daughter (Aiza) eyes bab.

Table of Contents

Introduction Générale 1

1- Literature Review 4

1.1 Fungi and secondary metabolites 4

1.2 *Aspergillus ochraceus* / *Aspergillus westerdijkiae* NRRL 3174 11

1.2.1 Ochratoxin A 13

1.2.2 Penicillic acid 16

1.2.3 Methylsalicylic acid, asperlactone and isoasperlactone 18

1.2.4 Mellein and Hydroxymellein 21

1.3 Fungal polyketides synthases 23

1.3.1 PKS producing reduced polyketides 27

1.3.2 PKS producing partially reduced polyketides 27

1.3.3 PKS producing non-reduced polyketides 28

1.4 Architectur base classification of PKSs 29

1.5 Nonribosomal Peptide Synthetases 31

1.6 Hybrid between PKS and NRPS 36

1.7 Biosynthetic pathways of different fungal polyketide metabolites 37

1.7.1 Biosynthetic pathway of OTA 37

1.7.2 Biosynthetic pathway of aflatoxin B1 39

1.7.3 Biosynthetic pathway of Lovastatin 42

1.7.4 Biosynthetic pathway of Patulin 45

1.8 Organization of polyketide gene clusters 47

2- Materials and Methods 53

2.1 Materials 53

2.1.1 Liste of kits utilized 53

2.1.2 List of products utilised 54

2.1.3 Apparatus used 56
2.1.4 Culture medium 57
a. Czapek Yeast Extract Agar (CYA) 57
b. Czapek Dox Agar (CZ) 58
c. Synthetic Medium (SM) 58
d. Malt Extract Agar (MEA) 59
e. Yeast Extract Saccharose (YES) 59
f. LB Broth 59
2.1.5 Oligonucleotide primers used 60
2.2 Methodology 61
2.2.1 Inoculums preparation and conservation 61
2.2.2 Extraction of ochratoxin A and other fungal secondary metabolites from solid culture medium 62
2.2.3 Extraction of ochratoxin A and other fungal secondary metabolites from liquid culture medium 62
2.2.4 Fluorescence and UV based HPLC Analyse 62
2.2.5 Fungal nucleic acid extraction 63
a. Preparation of fungal material 63
b. Genomic DNA extraction 63
i. High molecular weight DNA extraction 64
ii. CTAB method 64
iii. Quick method of DNA extraction 65
c. Total RNA extraction 65
d. Quantification of DNA and RNA 66
2.2.6 Horizental agarose gel electrophoresis 66
a. Gel preparation and migration 67
b. Vizualisation of the gel after electrophoresis 67
2.2.7 PCR (polymerase chain reaction) 67
2.2.8 Reverse transcription –PCR 68
2.2.9 Cloning 69
a. Principal of cloning 69
b. Preparation of Petri dishes containing LB agar 69
c. Cloning protocol 70
d. Ligation 70
e. Transformation 70
f. Analysis and conservation of clones 70
g. Mini preparation of plasmid DNA 71
2.2.10 Transformation vectors formation and transformation of *A. westerdijkiae* .. 71

a. Formation of transformation vector by classical method 71
b. Formation of transformation vector by modified double joint PCR..... 72
c. Complementation vector formation 74
d. Formation of protoplasts 74
e. Purification of protoplasts..... 75
f. Transformation..... 75
2.2.11 Confirmation of gene disruption and analyses of the mutants through PCR and Southern hybridization 76

Southern hybridization..... 77
a. Restriction digestion of genomic DNA..... 77
b. Southern transfer to Nylon membrane 77
c. Prehybridization 79
d. Radioactive labeling of probe 79
e. Hybridization 80
f. Wash 81
g. Detection 81

3- Results and Discussion 82

3.1 *Aspergillus westerdijkiae* polyketide synthase gene "*aoks1*" is involved in the biosynthesis of ochratoxin A. 85

Abstract 86

1. Introduction..... 87

2. Materials and methods 89

3. Results and Discussion 94

References 98

3.2 A polyketide synthase gene "*aolc35-12*" controls the differential expression of ochratoxin A gene "*aoks1*" in *Aspergillus westerdijkiae* 113

Abstract 114

1. Introduction..... 115

2. Materials and Methods..... 117

3. Results and discussion 122

References 125

3.3 Cloning and characterization of novel methylsalicylic acid synthase gene involved in the biosynthesis of isoasperlactone and asperlactone in *Aspergillus westerdijkiae* 136

Abstract 137

1. Introduction 138

2. Materials and Methods 140

3. Results 145

4. Discussion 148

References 151

3.4 A polyketide synthase – non ribosomal peptide synthase gene deferentially controls penicillic acid, isoasperlactone and asperlactone genes in *Aspergillus westerdijkiae* 162

Abstract 163

Introduction 164

Materials and Methods 166

Results 171

Discussion 174

References 177

4- General discussion and Perspectives 191

4.1 General discussion 191

4.2 Perspectives 195

References 197

List of abrevations

ACP:	Acyl Carrier Protein
AT:	Acyl transferase
Blast:	Basic Local Alignment Search Tool
bp:	Base pair
cDNA:	Complementary desoxyribonucleic acid
CoA:	Coenzyme-A
DNA:	Desoxyribonucleic acid
DH:	Dehydratase
ER:	Enoyl reductase
Etc:	et cetera
FAS:	Fatty Acid Synthase
HPLC:	High performance liquid chromatography
KR:	Ketoreductase
KS:	Ketosynthase
MSA:	Methylsalicylic acid
MSAS:	Methylsalicylic acid synthase
MT:	Methyltransferase
NRPS:	Non-ribosomal peptide synthase
OD:	Optical Density
OTA:	Ochratoxine A
PCR:	Polymerase chain reaction
PKS:	Polyketide synthase
Q-PCR:	Quantitative PCR
RT-PCR:	Reverse Transcription PCR
RNA:	Ribonucleic acid
TE:	Thioesterase
UV:	Ultraviolet

List of publications and communications

PUBLICATIONS

1. Bacha, N., Atoui, A., Mathieu, F., Liboz, T., Lebrihi A., 2009. *Aspergillus westerdijkiae* polyketide synthase gene "aoks1" is involved in the biosynthesis of ochratoxin A. Fungal Genetics and Biology 46, 77-84.

2. Bacha, N., Dao, H.P., Atoui, A., Mathieu, F., O'Callaghan, J, Puel, O., Liboz, T., Dobson, D.W., Lebrihi A., 2009. Cloning and characterization of novel methylsalicylic acid synthase gene involved in the biosynthesis of isoasperlactone and asperlactone in *Aspergillus westerdijkiae*. Fungal Genetics and Biology 46, 742-749.

3. Bacha, N., Mathieu, F., Liboz, T., Lebrihi A., A polyketide synthase – non ribosomal peptide synthase gene differentially controls penicillic acid, isoasperlactone and asperlactone genes in Aspergillus westerdijkiae (under consideration in Microbial biotechnology).

4. Bacha, N., Mathieu, F., Liboz, T., Lebrihi A., A polyketide synthase gene "*aolc35-12*" controls the differential expression of ochratoxin A gene "*aoks1*" in *Aspergillus westerdijkiae* (under preparation)

INTERNATIONAL CONFERENCES PROCEEDINGS

The AMMI Canada - CACMID Annual Conference, Vancouver (February 27 - March 1, 2008)

Bacha, N., Mathieu, F., Lebrihi A., Characterization of two polyketide synthase genes involved in the biosynthesis of penicillic acid, Asperlactone and Isoasperlactone in Aspergillus ochraceus NRRL 3174.

7th Congrès National de la Societe Francaise de Microbiologie Nantes France (30 mai – June 1, 2007)

Atoui, A., Bacha, N., Mathieu, F., Lebrihi A., Characterization of a polyketide synthase gene involved in the biosynthesis of lactone metabolites in *Aspergillus ochraceus* NRRL 3174.

POSTER PRESENTATION

International Society for Mycotoxicology Annual Conference 2009 Vienna Austria

Bacha, N., Mathieu, F., Liboz, T., Lebrihi A. Characterization of various PKS genes included in secondary metabolites biosynthesis pathway in *Aspergillus westerdijkiae* NRRL 3174.

7th Congrès National de la Societe Francaise de Microbiologie Nantes France (30 mai – 1 June 2007)

Bacha, N., Mathieu, F., Lebrihi A., Characterization of the second polyketide synthase gene involved in the biosynthesis of ochratoxin A in *Aspergillus ochraceus* NRRL 3174.

Introduction Générale

Introduction Générale

Dans les changements mondiaux actuels, les mesures préventives et sécuritaires pour la santé humaine sont un besoin fondamental. La sécurité sanitaire des aliments est devenue un souci international majeur ces dernières années, la contamination microbiologique et chimique est extrêmement préoccupante, les risques chimiques résident dans la contamination de l'alimentation humaine et animale par les mycotoxines (les métabolites toxiques de champignons). Ces molécules représentent actuellement un important problème de sécurité sanitaire des aliments.

La plupart des mycotoxines appartiennent au groupe polycétones de metabolites secondaires (Doull *et al.*, 1993; Kawaguchi *et al.*, 1988). *Aspergillus* et *Penicillium* sont les espèces le plus abondamment étudiées pour la biosynthèse de plusieurs polyketides toxiques et pharmacologiques (Nancy *et al.*, 1994) incluant l'aflatoxine, l'ochratoxine A (OTA), la patuline, l'acide methylesalicylique, l'acide penicillique, la melleine, l'aspyrone, l'asperlactone, l'isoasperlactone et la penicillin. La diversité chimique et les utilisations potentielles de ces composés sont pratiquement illimitées (Panagiotou *et al.*, 2009) .

Dans le 11ième siècle une pathologie provoquée par l'ergot de seigle (*Claviceps purpurea*) a été découverte en France, c'est seulement en 1960 que l'aflatoxine a été mise évidence en Grande-Bretagne ouvrant la voie à la découverte d'autres mycotoxines.

Certaines mycotoxines comme les aflatoxines, l'OTA et la patuline ont été largement étudiées et réglementées tandis que d'autres comme acide penicillique et sterigmatocystin restent moins bien connues. La mondialisation des échanges a compliqué la façon dont nous traitons les mycotoxines dans les normes que les organismes de réglementation deviennent souvent monnaie d'échange dans les négociations commerciales. Si les pays développés ont bien développé les infrastructures pour le suivi et le respect des normes de qualité des aliments, les pays en développement ne sont pas encore protégés contre les aliments contaminés. Selon la FAO au moins 25% des cultures alimentaires sont contaminées par les mycotoxines au moment où la production agricole des produits de base soutient à peine la croissance de la population mondiale (Boutrif & Canet, 1998).

Parmi les polycétones certaines sont toxiques, alors que d'autres jouent un rôle pharmacologique très important (Doull *et al.*, 1993; Kawaguchi *et al.*, 1988). La lovastatine est un métabolite de type polycétone avec de puissant effets hypocholestérolémiant (Alberts *et al.*, 1980) et c'est un précurseur pour la synthèse de médicaments largement prescrits, la simvastatine (W. F. Hoffman *et al.*, 1986). Il existe d'autres polycétones comme la mellein et l'hydroxumellein qui ont été utilisées avec succès comme phytotoxine contre les plantes indésirables (mauvaises herbes) (Webb, 1991). L'acide Penicillique possède des activités antibactériennes contre les bactéries Gram (+) et Gram (-) et présente une haute activité herbicide (Madhyastha *et al.*, 1994; Michito *et al.*, 1996). C'est aussi un agent antifongique efficace, surtout contre *Phytophthra spp* (Kang & Kim, 2004). Deux autres metabolites secondaires fongiques l'isoasperlactone et l'asperlactone, sont des agents antimicrobiens efficaces (Rosenbrook & Carney, 1970; Torres *et al.*, 1998) et ont egalement des activités ovicidales contre *Nezara viridula* (Balcells et al., 1995; Balcells et al., 1998).

La biosynthèse des polycétones est catlysée par des groupes d'enzymes « polycétone synthase (PKS) » et « les hybrides entre la polycétone synthéase et les peptides synthetases non ribosomaux (PKS-NRPS)». Il existe plusieurs techniques comme les complementation chimiques, l'étude de RMN, l'incorporation des précurseurs marqués et les techniques de biologie moleculaire, qui ont été utilisés pour comprendre le mécanisme de biosynthèse de plusieurs polycétones (Harris & Mantle, 2001; Staunton & Sutkowski, 1991). Mais entre ces differentes techniques les techniques de biologie moleculaires ont permis l'identification et la caractérisation de plusieurs gènes de polycétones dans différents organismes (Atoui *et al.*, 2006; Kao *et al.*, 1994; McDaniel *et al.*, 1993; Puel *et al.*, 2007).

Aspergillus westerdijkiae est un champignon filamenteux qui est capable de produire plusieurs métabolites de type polycétone d'importance économique, y compris l'ochratoxine A, la mellein, l'hydroxymellein, l'acide penicilliaue, l'asperlactone et l'isoasperlactone. Récemment dans notre laboratoire, avec les techniques de biologie moleculaire plusieurs gènes de polycétones ont été identifié chez *Aspergillus westerdijkiae* et *Aspergillus carbonarius* (Atoui *et al.*, 2006) .

Les principaux objectifs de cette thèse sont ;

1. Cloner et caractériser les différents gènes des PKSs et de PKS-NRPS (polycétone synthase- peptide synthetases non ribosomaux) chez le champignon *A. westerdijkiae* NRRL 3174.

2. Identifier les différentes étapes dans les voies de la biosynthèse de l'ochratoxine A, de l'acide penicillique, de l'asperlactone et de l'isoasperlactone.

3. Chercher les différents intermédiaires et leur implication pour les productions de différentes polycétones.

4. Utiliser les gènes caractérisés pour la construction d'une Banque d'ADN génomique et identifier les différents clusters des gènes.

Cette étude permettrait dans le future l'expression des gènes hétérologues et l'obtention de souches non toxigéniques à partir des souches toxigéniques.

Literature review

1- Literature Review

1.1 Fungi and secondary metabolites

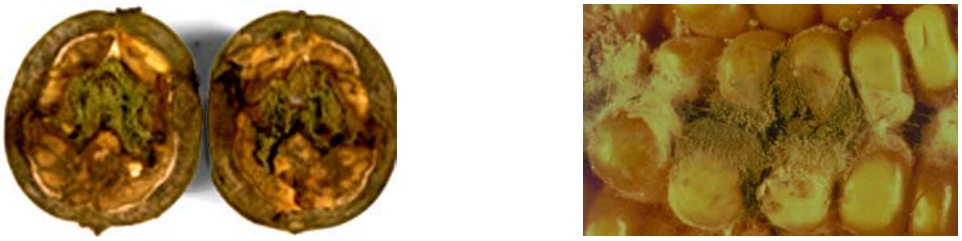
Fungi are a monophyletic group of microorganisms, also called the *Eumycota* (*true fungi* or *Eumycetes*). They live anywhere i.e. in the air, in water, on land, in soil, and in and on plants and animals. Within their varied natural habitats fungi usually are the primary decomposer organisms present. Many species are free-living saprobes (users of carbon fixed by other organisms) in woody substrates, soils, leaf litter, dead animals, and animal exudates. However, many other fungi are biotrophs, and in this role a number of successful groups form symbiotic associations with plants (including algae), animals (especially arthropods), and prokaryotes.

Fungi are important not only because they are equally distributed in our environment but they also offer enormous biodiversity, with around 70,000 known species, and an estimated 1.5 million species in total (Geoffrey, 2000). Most of these are filamentous fungi, which differ from the yeasts not only in their more complex morphology and development (e.g. asexual and sexual structures), but also in their greater metabolic complexity. In particular, they are known for production of secreted enzymes and secondary metabolites. Most of the secondary metabolites produced by fungi appear unnecessary for their primary functions. However, many metabolites appear indirectly important, influencing fungal growth and survival. The chemical characteristics of many secondary metabolites have been successfully exploited by man. At the beginning of the 21st century, fungi were involved in the industrial processing of more than 10 of the 20 most profitable products used in medicine. Two anti-cholesterol statins, the antibiotic penicillin and the immunosuppressant cyclosporin A produced by *Penicillium chrysogenum* and *Cephalosporium acremonium* respectively are among the top 10. Each of these has a turn over in excess of $1 billion annually (Anke & Thines, 2007; Wainwright, 1995). Some of the fungi are utilized in food industry e.g. *Penicillium roquefortii* and *P. camembertii*, for the production of cheese, several others are exploited for the production of different enzymes (40% of the enzymes are produced industrially), and organic acids (e.g. citric acid and gluconic acid produced by *Aspergillus* and

Penicillium species) (Carlile & Watkinson, 1997; Kiffer & Morelet, 1997; Perry *et al.*, 2004). About 22 % of the antibiotics identified are produced by filamentous fungi (Berdy, 1974; Strohl, 1997). Further several fungal species are considered to be effective as biocontrol agents. The fungal antagonists restrict the growth of plant pathogens by three suggested mechanisms: antibiosis, competition and parasitism. Besides, they also induce the defense responses in host plants, termed "induced systemic resistance" (van Loon *et al.*, 1998). Among the abovementioned mechanisms, antibiosis is considered the most important, in which the antagonists produce an array of secondary metabolites such as antibiotics and toxin, which contribute to the antagonistic activity of fungal biocontrol agents against plant pathogens (Mathivanan *et al.*, 2008).

The vast majority of the known fungal species are strict saprophytes, however, there are several fungal genera containing species that cause disease (e.g., infections, allergies, toxicity) in plants, animals and man. The undesirable presence of these fungi can change different aspects of food commodities, principally due to the production of different pigments e.g. dark pigment "melanin" produced by many aspergillus species, undesirable color change can occur (Figure 1a) (Butler & Day, 1998). Development of certain other fungi on food commodities can produce undesirable moldy structures and smells (Figure 1b). These changes can both affect the quality and quantity of food products and hence can cause reduction in food productions. At present crop loss due to invasive plant pathogens, especially fungi, is estimated at $21 billion per year only in the United States, greater than the loss caused by non-indigenous insects (Rossman, 2009).

Metabolites produced by fungi during their growth are also major cause of the impairment of foodstuff quality. These metabolites can not only changes the organoleptic properties (taste) of food, but can also produce serious threats to human and animal health e.g. risk of intoxication due the presence of mycotoxins (Table 1) (Steve & Gary, 1999). The appearance of human mycosis and allergies can be the result of fungal contaminated food consumptions. In some eastern countries of Europe, it has been shown that the endemic nephropathie of the Balkans (NEB) (renal cancer and cancer of urinary tract) is linked to the consumption of cereals contaminated with ochratoxin A (Krogh, 1987).



(a) **(b)**

Figure 1: Contamination of food by fungi: (a) *Aspergillus*-infected walnuts, (b) The fungus *Aspergillus* sporulating on corn.

Table 1. Some mycotoxins, fungi that produce them and symptoms in animals consuming feed contaminated with them (Steve & Gary, 1999).

Mycotoxin	**Fungi associated**	**Symptoms/toxicology**
Aflatoxin	*Aspergillus flavus, A. parasiticus*	liver necrosis, liver tumors, reduced growth, depressed immune response, carcinogen
Fumonisin	*Fusarium verticillioides, F. proliferatum*	Equine leukoencephalomalacia, porcine pulmonary edema
Trichothecenes and Deoxynivalenol	*F. graminearum, F. culmorum, F. poae F. sporotrichioides*	alimentary toxic aleukia, necrosis, hemorrhages, oral lesion in chickens, feed refusal, reduced weight gain
Ochratoxins	*P.verrucosum, A. ochraceus*	Porcine nephropathy; various symptoms in poultry
Citrinin	*Penicillium sp., Aspergillus sp.*	kidney damage
Cyclopiazonic acid	*Penicillium sp., Aspergillus sp.*	Neurotoxin
Sterigmatocystin	*Aspergillus sp.*	carcinogen, mutagen

Although reasons for why fungi produce secondary metabolites are not clear but it has been observed that these metabolites are commonly associated with sporulation processes in microorganisms (Hopwood, 1988; Mapleston *et al.*, 1992; Stone & Williams, 1992). Secondary metabolites associated with sporulation can be placed into three broad categories: (i) metabolites that activate sporulation (for example, the linoleic acid-derived compounds produced by *A. nidulans* (Calvo *et al.*, 2001; Calvo *et al.*, 2002; Champe & El-Zayat, 1989; Mazur *et al.*, 1991)), (ii) pigments required for sporulation structures, for example melanins required for the formation or integrity of both sexual and asexual spores and overwintering bodies (Alspaugh *et al.*, 1997; Kawamura *et al.*, 1999), and (iii) toxic metabolites secreted by growing colonies at the approximate time of sporulation, for example the biosynthesis of some deleterious natural products, such as mycotoxins (Hicks *et al.*, 1997; Trail *et al.*, 1995). These and other examples of secondary metabolites that fit into these categories are shown in table 2 (Bennett & Papa, 1988; Calvo *et al.*, 2001; Champe *et al.*, 1987; Champe & El-Zayat, 1989; Guzman-de-Pen˜a *et al.*, 1998; Mazur *et al.*, 1991; Takano *et al.*, 2000).

Fungal secondary metabolites are classified into several groups including polyketides (e.g. aflatoxin and fumonisins), non-ribosomal peptides (e.g. sirodesmin, peramine and siderophores such as ferricrocin), terpenes (e.g. T-2 toxin, deoxynivalenol (DON)), indole terpenes (e.g. paxilline and lolitrems) (Ellen & Barbara, 2008) (Figure 2). Among these groups, polyketides which are natural products biosynthesized by the polymerisation of acetyl and propionyl subunits in a similar process to fatty acid synthesis (James, 2008) are the largest group of metabolites occurring in their greatest number and variety. They all are originating from a polyketone, the polyketide chain, giving these substances their name.

Fungal polyketide metabolites are a major potential source of novel bioactive compounds. They are the building blocks for a broad range of natural products or are further derivatized. The wide spectrum of activity of polyketides makes them economically, clinically and industrially the most sought after molecules. *Ascomycetes* and the related “imperfect” fungi commonly known such as *Penicillium* and *Aspergillus*

are considered to be the large producers of polyketide secondary metabolites (Nancy *et al.*, 1994).

Table 2. Fungal secondary metabolites associated with sporulation processes and development (Calvo *et al.*, 2002).

Secondary metabolite	**Producing fungus**	**Association with development**
Linoleic-acid derived psi factor	*Aspergillus nidulans*	Induces sporulation; affects ratio of asexual to sexual spore development
Zearalenone	*Fusarium graminearum*	Induces sporulation; enhances perithecial formation
Butyrolactone I	*Aspergillus terreus*	Induces sporulation and lovastatin production
Melanin	*Colletotrichum lagenarium*	Associated with appressorial formation
Melanin	*Alternaria alternate*	UV protection of spore
Melanin	*Cochliobolus heterotrophus*	Required for spore survival
Spore pigment	*Aspergillus fumigatus*	Required for virulence
mycotoxins	*Aspergillus spp.*	Produced after sporulation

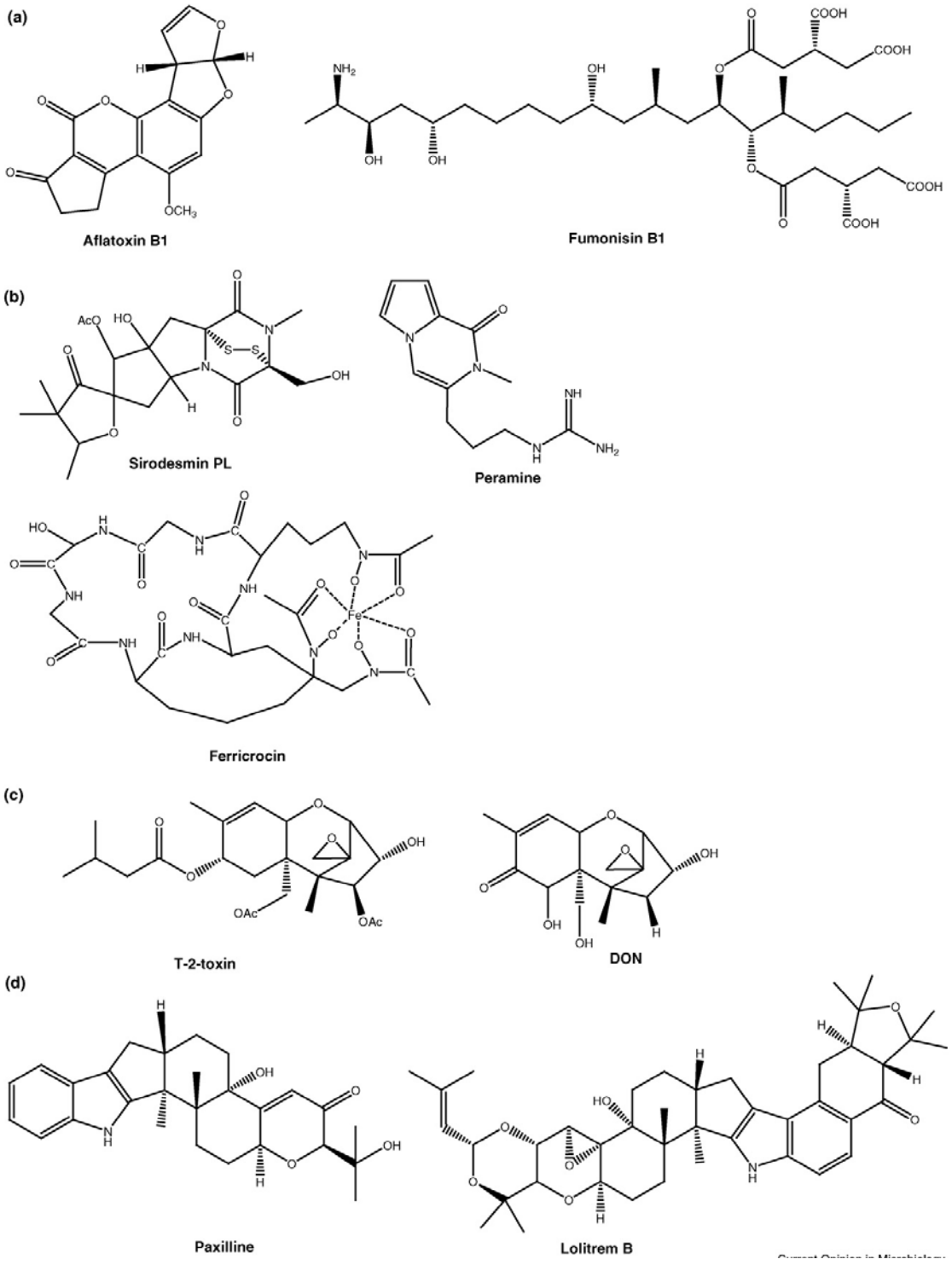


Figure 2: Different classes of fungal secondary metabolites: (a) polyketides, (b) non-ribosomal peptides, (c) terpenes, and (d) indole terpenes.

Polyketide metabolites present a remarkable diversity in their structure (Figure.3). Varying from the simplest tetraketides such as 6-methylsalicilic acid (1, 6-MSA) and orsellinic acid (2) to highly modified aflatoxins (5) (Isao *et al.*, 1998). Fungal polyketides are mostly aromatic compounds, though reduced complex type polyketides patuolide (6), brefeldin (7), lovastatin (8) and related compounds are not uncommon in fungi.

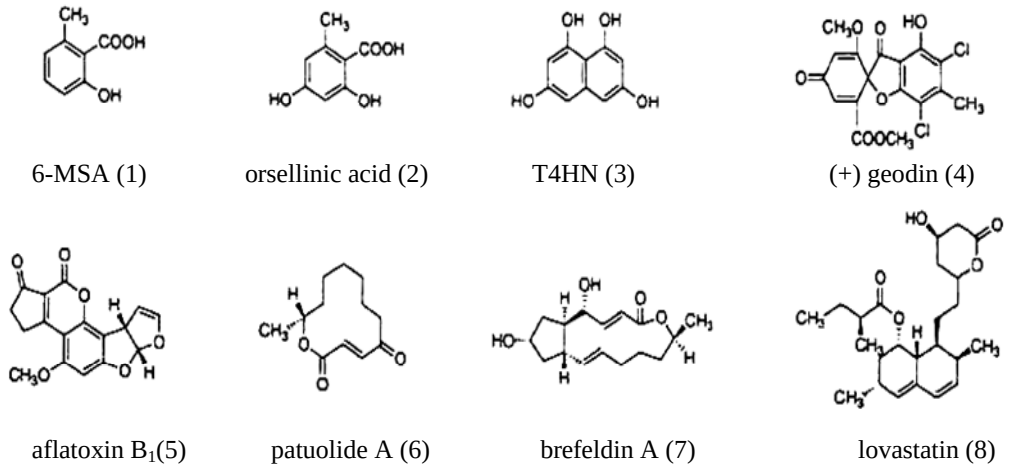


Figure 3. Diversity in the chemical structure of polyketides.

Fungal polyketides are a very diverse family of natural products with diverse biological activities and pharmacological properties. Several are known as mycotoxins like aflatoxin, ochratoxin, zearalenon, citrinin, patuline fumonisines, penicillic acid etc (Reboux, 2006). Several others are pharmacologically important like the antihypercholestemic drug lovastatin and its derivatives, the immunosuppressant cyclosporin, and ergotamine (Mathias & Dirk, 2007), the anti-fungal drugs echinocandins (Nyfelder & Keller, 1974) and antivirals i.e. HIV-integrase inhibitors, integrasone and integric acid (Marchand *et al.*, 2003). The polyketide metabolites asperlactone and isoasperlactone have both insecticidal and weedicidal effects (Balcells *et al.*, 1995; Balcells *et al.*, 1998).

1.2 *Aspergillus ochraceus* / *Aspergillus westerdijkiae* NRRL 3174

Aspergillus ochraceus NRRL 3174 also known as Wilhelm strain (Wilhelm, 1887) belongs to the *Aspergillus* section Circumdati (also known as *A. ochraceus* group of Raper & Fennell (Raper & Fennell, 1965)), class Deuteromycetes (imperfect fungi, reproducing asexually), Ordre Hypomycetes, and family Moniliaceae. This group of organisms is especially well known for its production of ochratoxin A, named after the producer *A. ochraceus* CBS 263.67 / NRRL 3174 (Van der Merwe *et al.*, 1965) and has attracted much interest by its role in the ochratoxin A contamination of coffee (Frisvad *et al.*, 2004b; Sua´rez-Quiroz *et al.*, 2004). Interestingly *A. ochraceus* NRRL 3174 or Wilhelm strain is now re-identified as *Aspergillus westerdijkiae*[1] (Frisvad *et al.*, 2004a). Hence several species previously identified as *A. ochraceus* could now be effectively named *A. westerdijkiae*. This fungus has got its name in honour of a former director of the CBS, Prof. Dr Johanna Westerdijk.

After 7 days of incubation on CYA (Czapek Yeast Agar) medium at 25 °C colony of *A. westerdijkiae* NRRL 3174 attains a diameter of 49-57 mm, while in MEA (Malt agar extract) its colony could be 42-47 mm in diameter, but never grows in CYA at 37°C. Cultural characteristics of this fungus include: production of good conidia on CYA at 25°C, coloring pale to light to dull yellow (color code:3B3–3A3); mycelium white, inconspicuous; sclerotia sparsely produced; pale yellow after 7 day, becoming dull orange at age. Reverse crème brown, no soluble pigment present. Good sporulation on MEA, velvety, pale to light or dull yellow (3A3–3B3) after 7 day; mycelium white, sclerotia sparsely formed, overgrown by conidial state and in shades of orange, reverse brown centre with yellow to medium–colored edge (Figure 4) (Frisvad *et al.*, 2004a) (http://www.aspergillus.org.uk/indexhome.htm?secure/speciesdatabase/westerdijkiae.php~main) . *A. westerdijkiae* is morphologically similar to *Aspergillus ochraceus*, though it is unable to grow at 37 °C. The white to cream sclerotia produced by *A. westerdijkiae* differ from the pink to vinaceous purple sclerotia of *A. ochraceus*.

[1] *Aspergilus westerdijkiae* and *Aspergillus ochraceus* NRRL 3174 are same (Frisvad *et al.*, 2004a). Hence in this thesis information about *A. ochraceus* NRRL 3174 are considered equally for *A. westerdijkiae*.

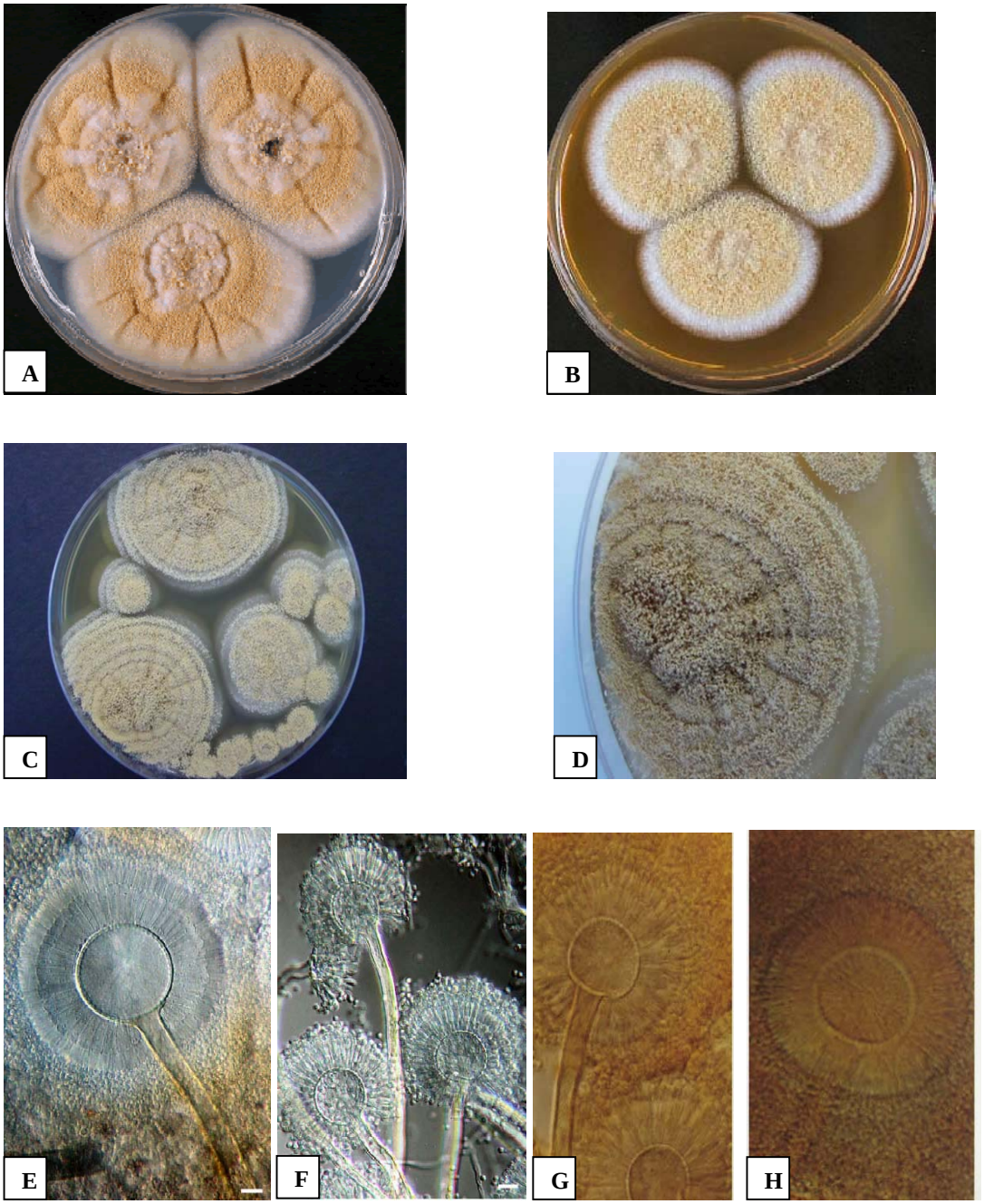


Figure 4. *Aspergillus westerdijkiae* after 7 days of incubation at 25 °C in CYA (A) and MAE **(B)**. Conidiophores **(E, F)**. (Frisvad *et al.*, 2004a).

Aspergillus ochraceus after 7 days of incubation at 25 °C in MAE (C and D). Conidiophores (G, H).

A. westerdijkiae is xerophile filamentous fungi hugely found in our environment. This fungus is frequently isolated from decomposed vegetables, soil, and desert. *A. westerdijkiae* principally causes seed and grain rot especially in maize. It contaminates many agriculture products including cereals, coffee, grapes (Christensen & Tuthill, 1985; Kozakiewicz, 1989). As this fungus best grows in temperature range of 15-31°C with water activity of 0.79, so most of the word regions favour the fungus to grow.

Aspergillus westerdijkiae has double economic interest. This fungus is used in food industry for the bioconversion of steroids and alcaloides (Chen, 1994; Richard *et al.*, 1983) and also in the production of various enzymes (Chadha & Garcha, 1992). *A. westerdijkiae* is capable of producing many secondary metabolites including ochratoxin A & B, penicillic acid, mellein, 4-hydroxymellein, xanthomegnin, viomellein, vioxanthin, circumdatins (A–G), asperlactone, isoasperlactone, asperloxins and aspergamides (=avrainvillamides = stephacidins) etc (Ciegler *et al.*, 1972; Robbers *et al.*, 1978; Stack & Mislivec, 1978; Van der Merwe *et al.*, 1965). The partially characterised extrolite NB1 is produced by all isolates of *A.westerdijkiae*, but not produced by any strain of *A. ochraceus.*

Details of some of the economically important polyketide metabolites produced by *A. westerdijkiae* are given bellow;

1.2.1 Ochratoxin A

The family of ochratoxin (Chemical formula $C_2OH_{18}ClNO_6$ and Molecular weight 403.8 g/mol) contains a dozen of known molecules, but its most important representative is ochratoxin A (OTA) ((5-Chloro- 3,4-dihydro- 8-hydroxy- 3-methyl- 1-oxo- 1H-2-benzopyran-7-yl) –carbonyl)- *L*- phenylalanine) (Figure 5). OTA is consisting of a dihydroisocomurin moiety (the pentaketide-derived ochratoxine α) linked through thr carboxyl group to phenylalanine. Corresponding des-chloro analogues are ochratoxine B (OTB) and β. OTA is a toxic metabolite produced primarily by *Aspergillus*, but also by *Penicillium* and other molds in a divers array of environmental conditions. It is a white crystalline powder. Recrystallized from xylene, it forms crystals that emit green (acid

solution) and blue (alkaline solution) fluorescence in ultraviolet light; the melting point of these crystals is 169ºC (IARC, 1993). Infact the optimal temperature for the production of OTA by *A. westerdijkiae* is 28 °C, bellow 15 °C and above 37 °C its production is highly reduced. In contrary *Penicillium viridicatum* can produce OTA in a divers range of temperatures between 4 to 30 °C. Hence in cold regions OTA is mostly produced by *Penicillium sp.*, while in hot regions it is mostly *Aspergillus sp.* producing it (Pohland *et al.*, 1992; Varga *et al.*, 1996).

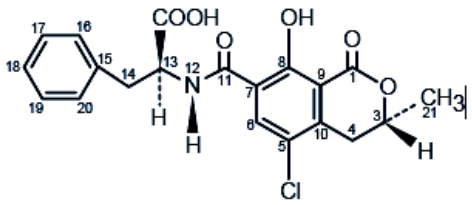


Figure 5. Chemical structure of ochratoxine A.

Ochratoxine A is principally found in cereals (wheat, corn, rye, barley, oats), but also can be found in rice, soya, coffee, cocoa, beans, peas, peanuts fresh grapes and dry fruits (figs, grapes). It has also been detected in various cereal based product like flour, bread and pasta (Majerus *et al.*, 1993), in beer (El-Dessouki, 1992) and even in wine and in grape juices (Zimmerli & Dick, 1996). Grains stored under high moisture/humidity (> 14 %) at warm temperatures (> 20 °C) and / or inadequately dried potentially can become contaminated by OTA (Ominski *et al.*, 1994). Damage to the grain by mechanical means, physical means or insects can provide a portal of entry for the OTA producing fungus. Initial growth of fungi in grains can form sufficient moisture from metabolism to allow for further growth and mycotoxin formation.

Ochratoxin A is primarily a kidney toxin but in sufficiently high concentrations it can damage the liver as well. OTA is reasonably anticipated to be a class B animal and human carcinogen based on sufficient evidence of carcinogenicity in experimental animals. When ochratoxin A was administered in the diet, hepatocellular tumors (designated as well-differentiated trabecular adenomas), renalcell tumors (renal cystadenomas and solid renal-cell tumors), hepatomas (some exhibiting the trabecular

structure), and hyperplastic hepatic nodules were observed in male mice. In another study, administration of ochratoxin A in the diet induced hepatocellular carcinomas and adenomas in female mice. Gavage administration of ochratoxin A to male and female rats resulted in a dose-related increase in the incidence of renal-cell adenomas and adenocarcinomas; further, metastasis of the renal-cell tumors was also observed in male and female rats. When administered by gavage, ochratoxin A increased the incidence and multiplicity of fibroadenomas of the mammary gland in female rats (IARC, 1993; Ribelin *et al.*, 1978).

The occurance of OTA in various foodstuffs is shown in table 3 (Magan & Olsen, 2004). These data confirm that OTA is found in a wide range of food stuffs from many different countries and in varying concentration. In a total diet study in the UK, low concentration of OTA was detected in all 50 samples analysed. In this study, OTA levels have been

Table 3. Occurance of OTA in selected foodstuff (Magan & Olsen, 2004)

Food stuff	**Origin**	**Concentration (µg/kg)**	**Contaminated samples**
wheat	Germany 1997	0.6-0.7	2/14
wheat	Germany 1998	0.6-0.8	7/29
wheat	Hungary	0.12-0.5	3/36
Wheat flour	Hungary	0.11-0.15	2/16
Maiz meal	Hungary	0.4	1/1
Oats	Norway	0.065-0.47	3/22
Beer	South Africa	3-3340	10/32
Beer	Europ	0.025-0.121	40/40
Pure roasted coffee	Hungary	0.17-1.3	33/50
Coffee products	Europe	0.9	299/933
Chocolate	UK	0.16	30/40
Cocoa powder	UK	1.67	20/20

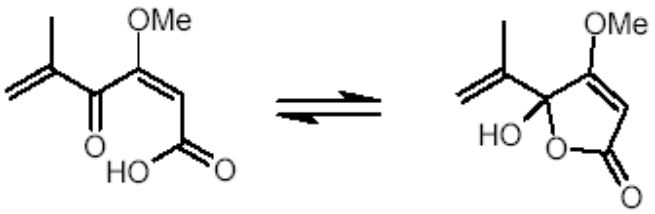
Table 4. European Union regulation for ochratoxin (μg/kg) (FAO Food and Nutrition Paper No 81, 2004)

Product	**Concentration**
Raw cereal grains	5
Products derived from cereals intended for direct human consumption	3
Dried vine fruit (currants, raisin and sultanas)	10

shown to varry between different commodities and withen different batches of commodities. Regulations for ochratoxin A are present in the European Community (FAO Food and Nutrition Paper No 81, 2004) (Table 4).

1.2.2 Penicillic acid

Penicillic acid (ϒ-keto-β-methoxy-∂-methylene-A^{α}-hexenoic acid) is a α, β-unsaturated, five-member lactones and a typical mycotoxin produced by *Aspergillus sp.* and *Penicillium sp.* (Figure 6) (Korzybski *et al.*, 1978; Smith & Moss, 1985). This compound was first isolated in 1913 from *Penicillium puberulum* and subsequently from *P. cyclopium* by Raistrick who for the first time established its structure (James, 2008).



(a) (b)

Figure 6. Chemical structure of penicillic acid: (a) Ket-penicillic acid and (b) lactone-penicillic acid.

The potential carcinogenic nature of penicillic acid possibly makes it a cause for concern, however work that has reported elsewhere with *P. roqueforticell* M-247 has shown that these toxins may not be produced in proteinaceous substrates, such as cheese (Dickens & Jones, 1961). This mycotoxin is toxic in experimental animals and has also been reported to be carcinogenic. The cytotoxicity of penicillic acid was studied in rat alveolar macrophages (AM) *in vitro*. The effects of penicillic acid on membrane integrity were studied by measuring cell volume changes and 51Cr release (Sorenson & Simpson, 1986). The carcinogenic effect of PA was studied in combination with other toxins. A direct interaction of patulin and penicillic acid with cellular DNA has been consistently observed in appropriate bacterial and mammalian cell systems. Single and double strand breaks were induced by both toxins in HeLa and mammary carcinoma cell DNA. An approximately fourfold larger total dose of penicillic acid was used to achieve carcinogenic effects in rats and mice, similar to those obtained with patulin (Umeda *et al.*, 1977). PA also inhibit the Na± /K± ATPase (Na+, K+ activated ATPase) (Hayes, 1980), which catalyses the active transport of Na+ and K+ across the cell membrane.

Penicillic acid is mostly known for its toxicity to animals but some previous work showed the phytotoxic effect of this molecule. *P. cyclopium* and *P. canescens* are the main contaminant fungi detected in seeds harvested in the northwest of France. Such contaminants are fearsome, since they affect the seeds before harvest time and may find optimal developing conditions when the seeds are stored, leading to alteration of the germination quality of these seeds. It has been found that the altered germinative qualities of the seeds were solely due to the growth of contaminating fungi or to the related action of phytotoxic metabolites penicillic acid (Jacqueline & Daniel, 1985). This metabolite was further reported to inhibit the growth of young plant roots of rice (Sassa *et al.*, 1971) and of oat seedlings by lessening their respiration (Bastin & Roey, 1954). It was also reported to inhibit urease (Reiss, 1979) and RNase (Tashiro *et al.*, 1979) activities in experimental animals.

Hence penicillic acid should now be considered as a phytotoxin as are aflatoxin B1, rubratoxin B, and zearalenone (Brodnik, 1975). The toxicity of penicillic acid is about 5 % that of aflatoxin B1, which gave 50 % inhibition of seed germination at the dose of 25

µg / ml, whereas 500 µg of penicillic acid per ml were necessary for a 50 % decrease of the main root length. Nevertheless, penicillic acid occurs in such high quantities (up to 2% dry weight) in infected corn (Kurtzman & Ciegler, 1970) that it must be considered seriously. Several other factors increased the seriousness of penicillic acid as phytotoxin. About 50 % of *P. cyclopium* strains produce penicillic acid (Le Bars, 1979), and its biosynthesis is higher on broken kernels (Le Bars, 1980). Penicillic acid is stable in substrates with low water content (Le Bars, 1982) and accumulates at the low temperatures (Ciegler & Kurtzman, 1970) of typical storage conditions. There is no additional evidence for natural occurance of PA in foodstuffs since the earlier report of its occurance (up to 230 µg/kg) in commercial maize and beans (Magan & Olsen, 2004). An Indian survey of over 1000 samples of food grains was negative. This paucity of occurrence of data may be due to its instability e.g. it is reactive with glutathione.

1.2.3 Methylsalicylic acid, asperlactone and isoasperlactone

Methylsalicylic acid ($C_8H_8O_3$), is the archetypical tetraketide. It is a colourless or yellow to red oily liquid with characteristic smell (Forrester & Gaucher, 1972). Esters of methylsalicylic acid are largely used in the production of perfumes and food aroma under the name oil Wintergreen (http://fr.wikipedia.org/wiki/Salicylate_de_méthyle). Its smell is very strong and attractive. It is also known for its analgesic and antipyretic properties. Several esters of methylsalicylic acid can provoke the anti-insect-herbivores defens system in plants. If the plant is infested with herbivorous insects, the release such compound may function as an aid in the recruitment of beneficial insects to kill the herbivorous insects. Several other esters of methylsalicylic acid can be toxic when absorbed and can be even lethal when inters into the body system in large quantity. A dose of 5 mL (a coffee spoon) is possible to be fetal for a child (Tintinalli *et al.*, 2004). Apart from the toxic effect, this pheromon molecule has also been used to provoke successfully the salicylic acid based defence system in tobacco against tobacco mosaic virus (Vladimir *et al.*, 1997).

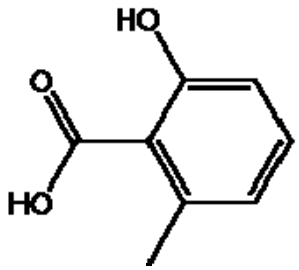


Figure 7. Chemical Structure of 6-methylsalicylic acid

Methylsalicylic acid is biosynthesized by many *Penicillium* and *Aspergillus* species including *A. westerdijkiae*. This substance was intimately involved in establishing the polyketide biosynthetic pathway (Bu'Lock *et al.*, 1968); it is one of the few fungal secondary metabolites whose enzymology has been examined successfully in detail (Dinroth *et al.*, 1976); it was the model system that Bu'Lock and his colleagues (1968) used to develop the trophophase (A period in culture production characterized by active microbial cell growth and the formation primary metabolites) / idiophase (A period in culture growth during which secondary metabolites are produced) description of secondary metabolism; and it is one of the limited number of fungal systems wherein it has been shown convincingly that induction of the metabolic enzymes in submerged liquid culture correlates with a medium nutrient depletion (Grootwassink & Gaucher, 1980). In view of the pivotal role that 6- methylsalicylic acid has played in the development of our knowledge of fungal secondary metabolism, it is clear that any general explanation of why this process exists and what function it serves must be validated in 6-methylsalicylic acid-producing systems.

Beside the direct role of 6-methylsalicylic acid, it is the key precursor / intermediate of several other important polyketides like mycotoxin patulin (James, 2008) and antibiotic avilamycin (Gaisser *et al.*, 1997). The importance of 6-methylsalicylic acid as precursor of patulin has been studied in various fungi. The first step in the production of patulin resides in the formation of 6-methylsalicylic acid, by the condensation of one molecule of acetyl-coA and 3 molecules of malonyl-coA. It has been shown that 6-methylsalicylic

acid specifically marked has been converted to patulin in *Penicillium patulum* (Bu'Lock, 1961; Tanenbaum & Bassett, 1958).

Asperlactone and isoasperlactone (9-chloro-8-hydroxy-8,9-deoxy) (Figure 8), along with patulin, isopatulin and orsellinic acid constitute a class of chemically related simples 5-membered cyclic lactones. The chemical structures of the two fungal metabolite i.e. asperlactone and isoasperlactone, have been deduced for the first time by the comparison of their spectroscopic data with that of aspyron and other known co-metabolites (Mary *et al.*, 1984). Both asperlactone and isoasperlactone have the same chemical structure but they are stereochemically different. Not much is known about the biosynthetic pathways of isoasperlactone and asperlactone, except for a hypothetical scheme proposed by James & Andrew (1991). According to this scheme, the isomeric metabolites aspyrone, isoasperlactone and asperlactone are derived from a common biosynthetic precursor, the diepoxide. James & Andrew (1991) further stated that asperlactone is formed directly from the diepoxide, while isoasperlactone is formed from diepoxide via aspyrone pathway. Asperlactone is biosynthesized from aspyrone by the decarboxylation and arrangements of the polyketide intermediates followed by stereospecific opening of epoxy circle (Brereton *et al.*, 1984).

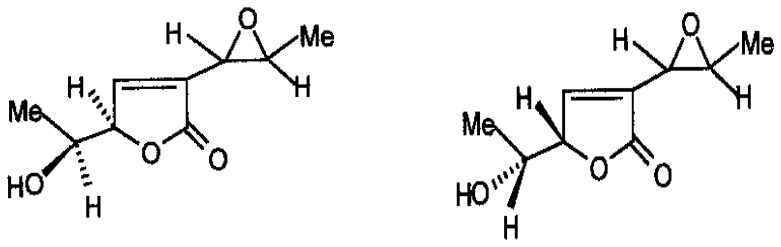


Figure 8. Chemical structures of (a) Asperlactone (b) Isoasperlactone

Asperlactone and isoasperlactone have been isolated for the first time from *Aspergillus* sp. in 1970 (Rosenbrook & Carney, 1970). It has been found that *A. melleus* is the key producer of both asperlactone and isoasperlactone (Garson *et al.*, 1984). It is only recently found that *A. ochraceus* (re-identified as *A. westerdijkiae*) is also an active producer of these metabolites (Awad *et al.*, 2005).

The activities of asperlactone and isoasperlactone are extensively studied in combination with other active polyketides metabolites like aspyrone and its synthetic derivatives. It has been found that asperlactone is more active than aspyrone (Balcells *et al.*, 1995). Asperlactone equally contains antibacterial and antifungal activities (Torres *et al.*, 1998). The bactericidal and fungicidal activities of asperlactone, isoasperlactone, aspyrone and some of its derivatives have been studied, with asperlactone and isoasperlactone in particular exhibiting strong activity against both bacteria and fungi; when compared to aspyrone derivatives (Balcells *et al.*, 1998). In addition asperlactone isolated from an *Aspergillus ochraceus* Wilhelm strain displayed insect growth regulator (IGR) activity against *Tribolium castaneum* (Herbst). When the synthetic derivatives of aspyrone were tested in combination with asperlactone and isoasperlactone for IGR and ovicidal activities an improved impact of the combination was observed against *T. castaneum* (Balcells *et al.*, 1998).

1.2.4 Mellein and Hydroxymellein

Recently, the isocoumarin compounds, mellein (3-methyl-8-hydroxy-3, 4-dihydroisocoumarin) and 4-hydroxymellein (3-methyl- 4, 8-dihydroxy-3, 4-dihydroisocoumarin) (Figure 9) were isolated from the culture medium of several *Aspergillus* species including the ochratoxin producing strain *A. westerdijkiae* (Cole et al., 1971; Sasaki et al., 1970).

Mellein and hydroxymellein are structurally similar to the dihydroisocoumarin moiety of OTA and they possibly have similar biological properties (Van der Merwe *et al.*, 1965). Because of this structural similarities with OTA, these metabolites were considered as intermediates of OTA (Huff & Hamilton, 1979). But later experiments with labelled

precursors of OTA ruled out this possibility and suggested that mellein and hydroxyl mellein are not intermediates in the biosynthetic pathway of OTA (Harris & Mantle, 2001).



Figure 9. Chemical structures of mellein and 4-hydroxymellein

The optimal biosynthetic conditions for mellein and hydroxyl-mellein have remained the topic of several studies in its principal producer *Aspergillus ochraceus* Wilhelm strain (Moor *et al.*, 1972). Moor *et al.*, (1972) demonstrated that mellein production showed a linear relationship with increasing sucrose concentration over the range studied, but production efficiency was best at 4, 8, and 16 %. 4-Hydroxymellein production efficiency was the same at sucrose concentrations of 2, 4, 8, and 16 %, similar to ochratoxin A and B production reported (Davis *et al.*, 1969). For both mellein and 4-hydroxymellein production a sucrose concentration of 8 % was optimal (based on total amount produced per flask). Evaluation of production on medium, with equimolar concentrations of 15 carbon source, revealed that these compounds which are oxidized normally through both the hexose monophosphate and glycolytic pathways supported both growth and metabolites production. The organism grew poorly on the tricarboxylic acid cycle intermediates and metabolite production was poor. L-Glutamic acid at 6 g / liter produced the maximal amounts of mellein and 4-hydroxymellein. Production of mellein and 4-hydroxymellein coincided with rapid mycelial growth at 20, 25, 30, and 35˚C. Incubation for longer than 8 to 10 days resulted in decreased yields of mellein but not 4-hydroxymellein. Incubation for 10 to 14 days at 25 to 30˚C was selected as optimal for production of both metabolites in the same medium (Moor *et al.*, 1972).

Mellein and hydroxymellein has been shown to posses an impressive array of biological activities (Sun & Toia, 1993). For example, mellein is one of the constituents of the mandibular secretion of Carpenter ants and the defensive secretion of termites. It occurs in the male hair pencil of the oriental fruit moth and has been shown to have pheromonal properties in the castes of *Camponotus pennsylvanicus.* Mellein, hydroxumellein or combination of these two has been successfully used as phytotoxine against several unwanted plants (weeds) (Webb, 1991). Sasaki et al. (1970) reported the LD50 of mellein and 4-hydroxymellein in intraperitoneally injected mice as 250 to 500 and 1,000 to 1,500 mg/kg, respectively.

1.3 Fungal polyketides synthases

In spite of the fact that polyketides are greatly diverse both in their structures and functions, they are biosynthesized through the same mechanism analogous to the biosynthesis of fatty acid (through the action of fatty acid synthase (FAS)). The key chain-building step of polyketide biosyhnthesis is a decarboxylative condensation analogous to the chain elongation step of classical fatty acid biosynthesis which occurs in a well-understood way described in Figure 10. Unlike fatty acid biosynthesis, however, in

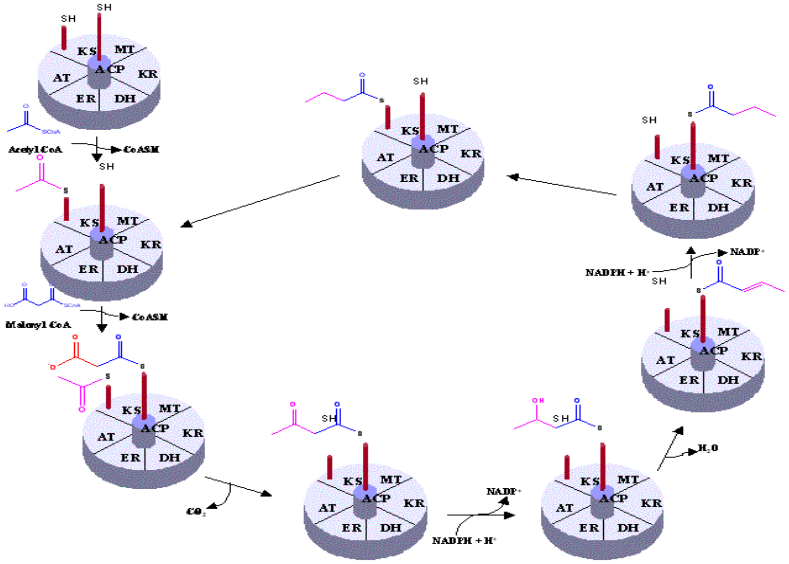


Figure 10. Classical chain elongation steps during fatty acid biosynthesis.

which each successive chain elongation step is followed by a fixed sequence of ketoreduction, dehydration and enoyl reduction, the individual chain elongation intermediates of polyketide biosynthesis undergo all, some, or none of these functional group modifications, resulting in a striking level of chemical complexity in the products (Figure 11). Additional degrees of complexity arise from the use of different starter units and chain elongation units as well as the generation of new stereo-isomers (Cox, 2000; Khosla *et al.*, 1999).

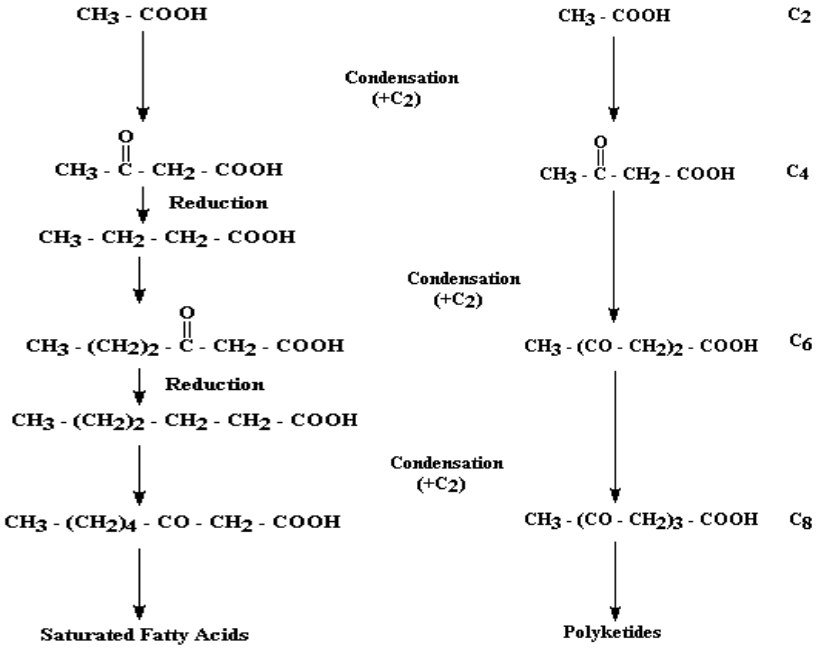


Figure 11. Comparison of the biosynthetic schemes of fatty acid and polyketide

Polyketides are synthesized by sequential reactions catalyzed by a collection of modular enzymes activities called polyketide synthases (PKSs), nonribosomal peptide synthetase (NRPS) and hybrid between polyketide synthase and nonribosomal peptide synthetase (PKS-NRPS) (Bentley & Bennett, 1999; Hopwood & Sherman, 1990; Sebastian *et al.*, 2007). These enzymes govern complex enzymology with fascinating mechanisms and great commercial appeal, because these enzymes create a multitude of secondary metabolites, many of which have become important drugs. These enzymes represent

some of the largest proteins known, and, as a consequence of their multifunctional character, a single protein can catalyze dozens of discrete biochemical reactions. For example, the rapamycin PKS (Schwecke *et al.*, 1995) and cyclosporin NRPS (Weber *et al.*, 1994) catalyze 51 and 40 steps in assembly of their respective products.

Polyketide synthases are large multi-enzyme protein complexes that contain a coordinated group of active sites or domains i.e. β-keto-acyl synthase (KS), acyl transferase (AT), ketoreductase (KR), dehydratase (DH), enoyle reductase (ER), methyl transferase (MT); acyle carrier protein (ACP) and thioesterase (TE). However AT, KS and ACP are the three principal domains while the remaing domains are optional (Figure 12).

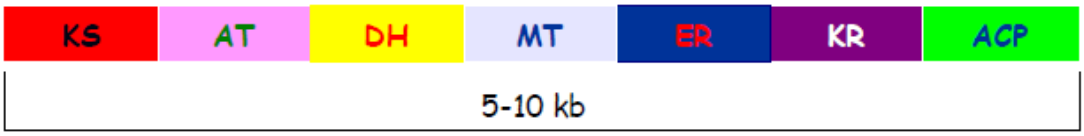


Figure 12. Structure of a typical polyketide synthase, showing different functional domains.

The PKS act in a step-wise manner to biosynthesize the corresponding polyketide 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 (Figure 13).

Different functional domains on a PKS play different functions during the biosynthesis of a polyketide (Gokhale & Tuteja, 2001). AT domain serves for the loading of starter, extender and intermediate acyl units; ACP domain hold the growing macrolide as a thiol ester; KS domain, which is the most conserved domain among different PKSs, catalyses chain extension; KR domain is responsible for the first reduction to an alochol functionality; DH domain eliminates water to give an unsaturated thiolester; ER domain catalyses the final reduction to full saturation; MT domain catalyses the incarporation of

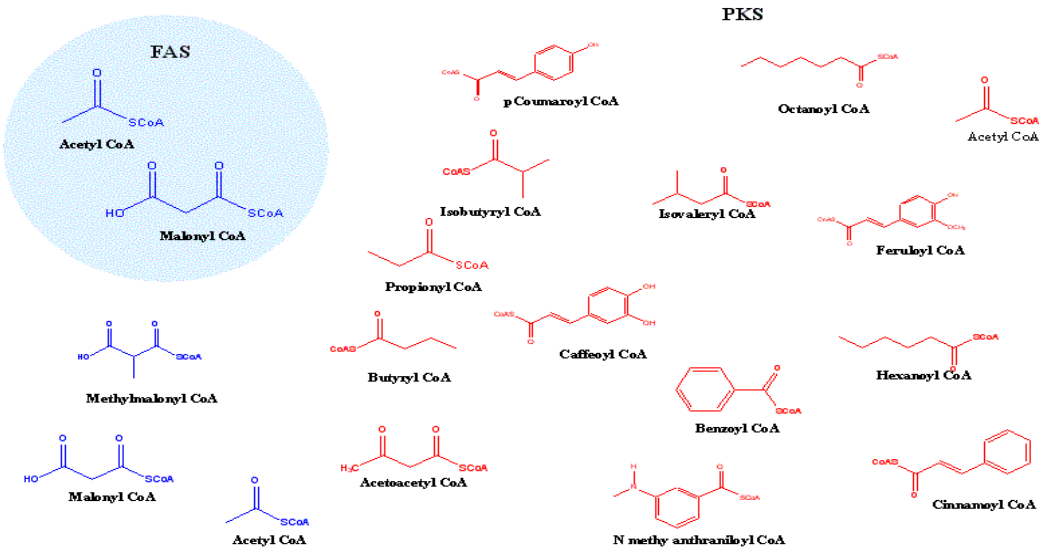


Figure 13. Different starter and extender units of polyketides.

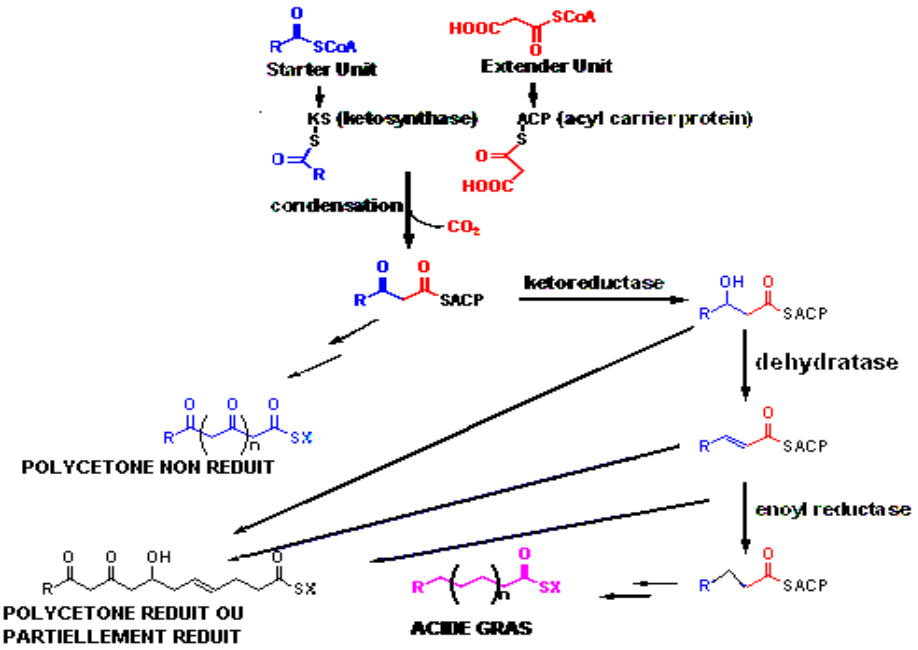


Figure 14. Chemistry of polyketide synthases: role of different functional domains (Gokhale & Tuteja, 2001).

methyle group; and finally a thiolesterase (TE) domain catalyses macrolide release and cyclisation (Figure 14).

In case of fatty acid biosynthesis, the ß-cetone group generated is completely reduced by a single sequential cycle of reduction (KR, DH and ER) before the following condensation until the skeleton attains the desired length. While this is not the case with PKSs, where the level of reduction varies by the partial or total omission of reduction reactions, which is the key to the diversity of fungal PKS (Fujii *et al.*, 2001).

Based on the level of reduction of polyketides, fungal PKSs are divided into three main classes (Figure 15), as described bellow;

1.3.1 PKS producing reduced polyketides

These are the PKSs which contains along with principal domains, all of the optional domains responsible for reduction reactions (KR, DH and ER). The polyketides generated by this group of PKS undergo several stages of reductions in their biosynthesis, so that all ß-keto grouping are almost reduced. Example of these PKSs are the nonaceton and diceton synthases of lovastatine (*A. terreus*) and compactine (*P. citrinum*), PKS of citrinine (*M. ruber*), PKS of fumonisine (*F. moniliform*) and PKS of T-toxine (*F. tricinctum*) etc.

1.3.2 PKS producing partially reduced polyketides

These PKSs contain KS, AT, DH, KR and ACP functional domains or where there is only one cycle of reduction leading to the partial reduction of polyketides. Several PKSs of this class have been characterized as methylsalicylic acid synthase (MSAS) important for the biosynthesis of patulin in *P. patulum* (Beck et al., 1990), and MSAS in *A. terreus* (Feng & Leonard, 1998).

1.3.3 PKS producing non-reduced polyketides

The PKSs in this class do not contain any domain of reduction (ER, DH ou KR), which explains the absence of any reduction activity. They can have certain additional domains of ACP and a domaine Claisen-type cyclase or C-terminus (Kroken *et al.*, 2003). This class includes the PKS of norsolinic acid, first intermediate in the biosynthetic pathway of aflatoxins in *A. flavus*, *A. parasiticus* and sterigmatocystine in *A. nidulans* (Feng & Leonard, 1998; Yu & Leonard, 1995), The PKS of wA type implied in the biosynthesis of spore pigments, YwA1 in A. *nidulans* and tetrahydroxynaphtalène synthase in the biosynthesis of melanin in *C. lagenarium* (Cox, 2000).

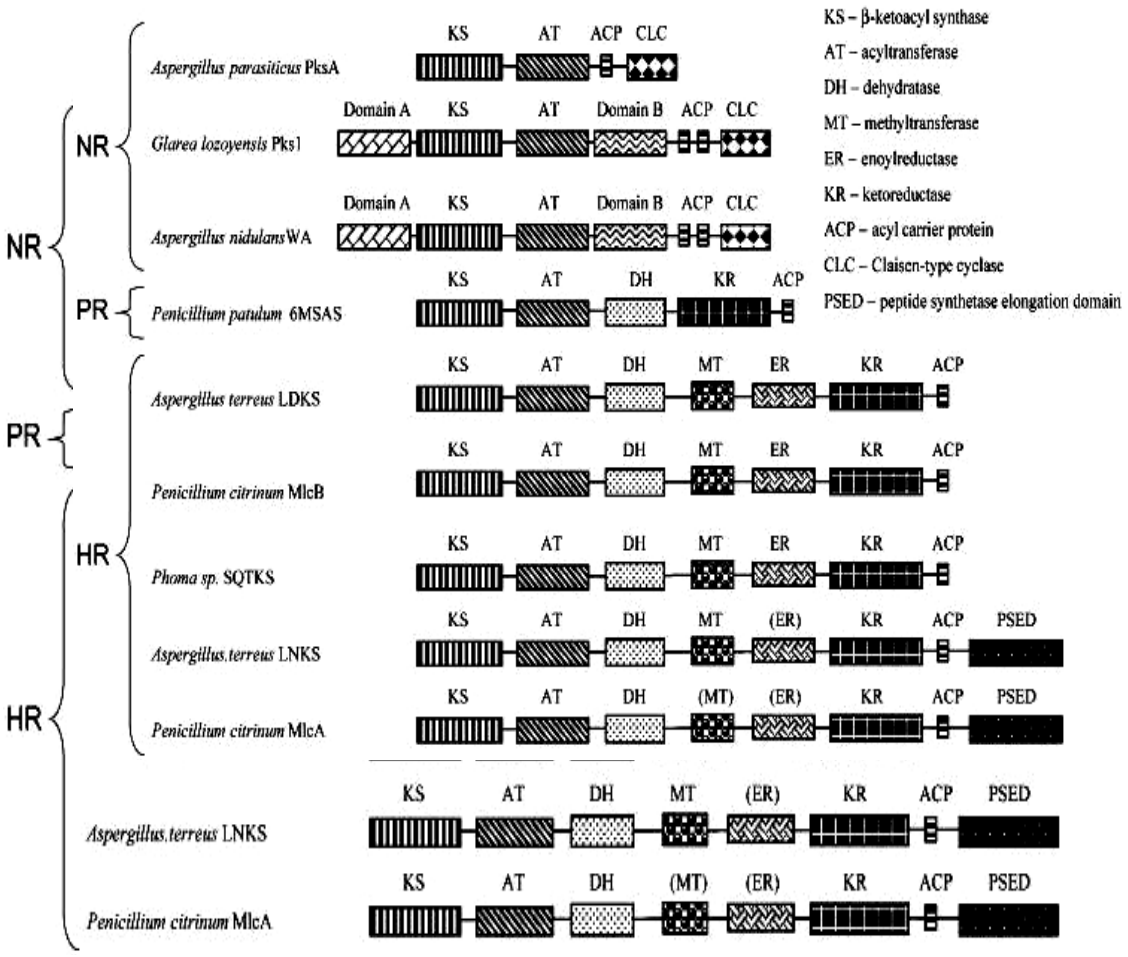
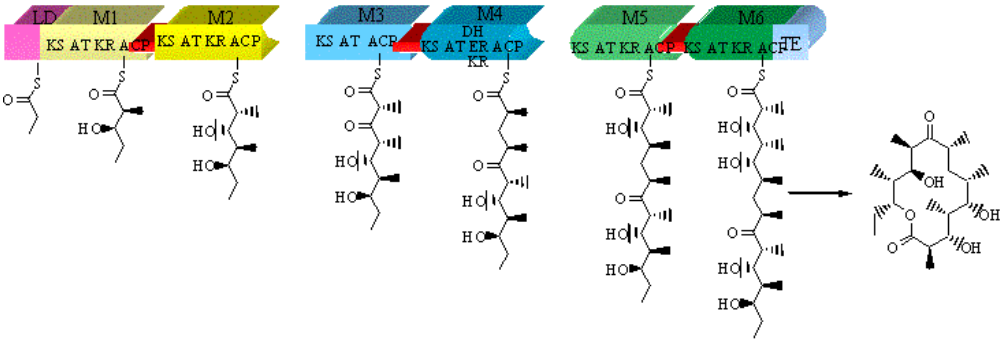


Figure 15: Organisation of functional domains diversity of fungal PKSs (NR : non reduced, PR : partially reduced and HR : highly reduced).

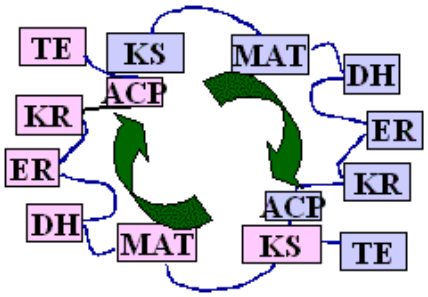
1.4 Architectur base classification of PKSs

At least three architecturally different PKS systems have been discovered in the microbial world (http://linux1.nii.res.in/~pksdb/polyketide.html). These systems are;

Type I systems consist of very large multifuctional proteins which can be either modular or processive (for example the unique modular systems responsible for synthesis of macrolides in bacteria like erythromycin, rapamycin, rifamycin etc. (Figure 16a)) or iterative (Figure 16b)(for example the lovastatin nonaketide synthase) (Gokhale & Tuteja, 2001). Iterative Type I synthases are analogous to vertebrate fatty acid synthases. These are typically involved in the biosynthesis of fungal polyketides such as 6-methylsalicylic acid and aflatoxin. These PKSs are large multidomain proteins carrying all the active sites required for polyketide biosynthesis.



(a)



(b)

Figure 16. Type I system of PKSs: (a) Modular and (b) iterative

The **iterative Type II** systems consist of complexes of mono-functional proteins exemplified by the actinorhodin PKS from *Streptomyces coelicolor*. In these synthases, active sites are distributed among several smaller, typically monofunctional polypeptides. Type II synthases catalyse the formation of compounds that require aromatization and cyclization, but not extensive reduction or reduction / dehydration cycles. These PKSs are analogous to bacterial Fatty Acid Synthases and are involved in the biosynthesis of bacterial aromatic natural products such as actinorhodin, tetracenomycin and doxorubicin. A hypothetical "dynamic" model of this system is presented in Figure 17.

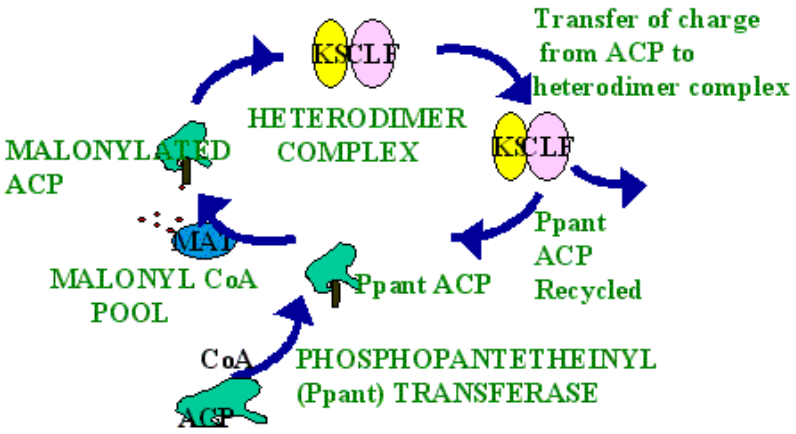


Figure 17. Iterative Type II system of PKSs.

Type III polyketide synthases are responsible for the synthesis of chalcones and stilbenes in plants and polyhydroxy phenols in bacteria. Chalcone synthase like proteins are comparatively small proteins with a single polypeptide chain and are involved in the biosynthesis of precursors for flavonoids (Figure 18). Unlike all other PKSs, these proteins do not have a phosphopantetheinyl (P-Pant) arm on which the growing polyketide chains are tethered.

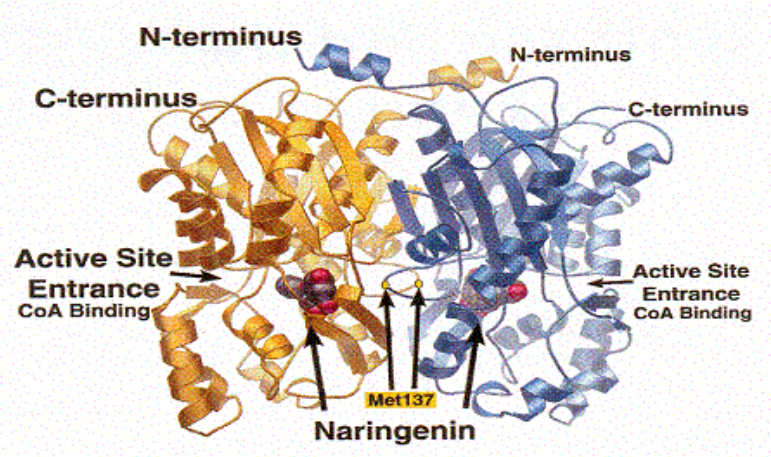


Figure 18. Representative model of type III polyketide synthases.

Most of the fungal PKSs are iterative type I. Recently, several chalcone synthase (CHS)-like genes PKS (belong to the type III PKS) have been discovered in some fungi like *A. oryzae*, *Neurospora crassa* et *F. graminearum* (Seshime et al., 2005).

1.5 Nonribosomal Peptide Synthetases

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 & Doehren, 1996; Schwarzer & Marahiel, 2001; Smith *et al.*, 1990).

NRPSs are organized into coordinated groups of active sites termed modules, in which each module is required for catalyzing one single cycle of product length elongation and modification of that functional group. The number and order of module and the type of domains present within a module on each NRPS determines the structural variation of the

resulting peptide product by dictating the number, order, choice of the amino acid to be incorporated and the modification associated with a particular type of elongation.

The minimum sets of domains required for an elongation cycle consist of a module. The modules are itself subdivided into different domains including Adenylation **(A)**, Thiolation **(T)** or Peptidyl Carrier Protein **(PCP)**, and Condensation **(C)** (Figure 19) (Schwarzer & Marahiel, 2001).

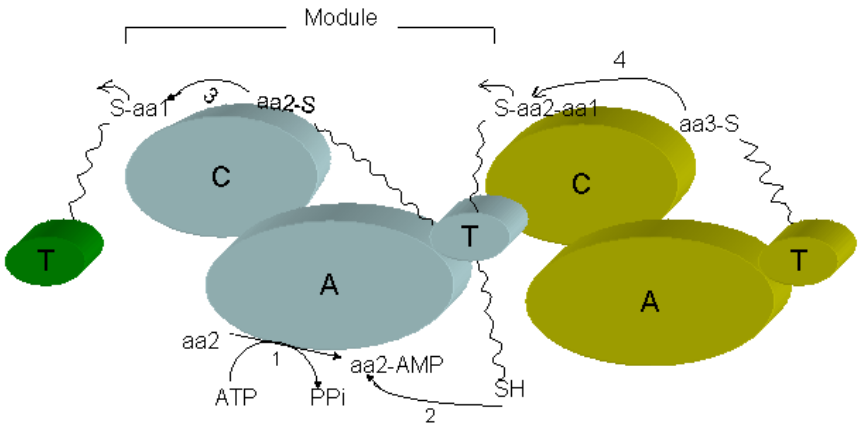


Figure 19. Minimum set of domains required in an NRPS

Each domain serves a specific function during the monomer incorporation. The A domain 500 to 600 amino acid residues is required for amino acid substrate recognition and activation. The 80 to 100 amino-acid-residue T domain, located downstream of the A domain, is the site for 4'-phosphopantetheine co-factor binding; the holoenzyme then activates aminoacyl substrates to form a thioester bond. A condensation domain (≈450 amino acids) is typically found after each A-T module and functions in peptide bond formation and elongation of the nascent peptide. Generally, the number and order of modules present in a nonribosomal peptide synthetase determine the length and structure of the resulting nonribosomal peptide. In addition to A, T, and C domains, an N-methyl transferase (M) domain that methylates the amino acid specified by the A domain may be inserted between the A and T domains of any given module, and an epimerase (E) domain that changes an amino acid from the L- to the D-form may be inserted between the T and C

domains. In some nonribosomal peptide synthetases, a thioesterase domain is found at the C-terminal end of the protein and is thought to release the nonribosomal peptide from the nonribosomal peptide synthetase (Cosmina *et al.*, 1993)

There exist three major peptide biosynthesis types through the involvement of NRPS. The first one is linear biosynthesis (Figure 20a). In this type of biosynthesis there is collinearity between the synthetase and the peptide product. This means that the modular sequences of synthetase corresponds to that of the monomers incorporated in the product. This type of biosynthesis is the one most frequently occured. The second type is iterative biosynthesis (Figure 20b). In this case, some modules of the synthetase are used more than once during the synthesis of a peptide. The last type is the non-linear biosynthesis (Figure 20c). In this type of biosynthesis, the arrangement of modules of synthetase is different from that of the monomer peptide.

All the multi-modular enzymatic assemblies are organized with a common molecular logic for natural product: acyl chain initiation, chain elongation, and chain termination, catalyzed by protein modules that pass the growing chain from N to C termini of the assembly lines (Figure 21). Monomer units, such as amino or carboxy acids for NRPS and acetate or propionate for PKS, are selected and installed as acyl / aminoacyl-S-enzyme intermediates on a carrier protein domain in each module. The reactive thiol group in each acyl carrier/peptidyl carrier protein domain is introduced as a 4′-phosphopantetheinyl arm by posttranslational priming by dedicated phosphopantetheinyl transferases (Walsh *et al.*, 1997). Specialized domains located within the corresponding NRPS/PKS modules can further modify the acylated substrates. Chain growth occurs as a series of translocating, elongating acyl/peptidyl-S-pantetheinyl carrier protein intermediates. The chain elongation chemistry in each module is by C-C bond formation in PKS assembly lines and by C-N amide bond formation in NRPS assembly lines, in upstream to downstream direction. When the full-length polyketide / polypeptide chain reaches the ultimate downstream carrier protein domain, the complete acyl chain is disconnected from its covalent thioester ether by a C-terminal thioesterase (TE) domain (Figure 21) (Bruner *et al.*, 2002).

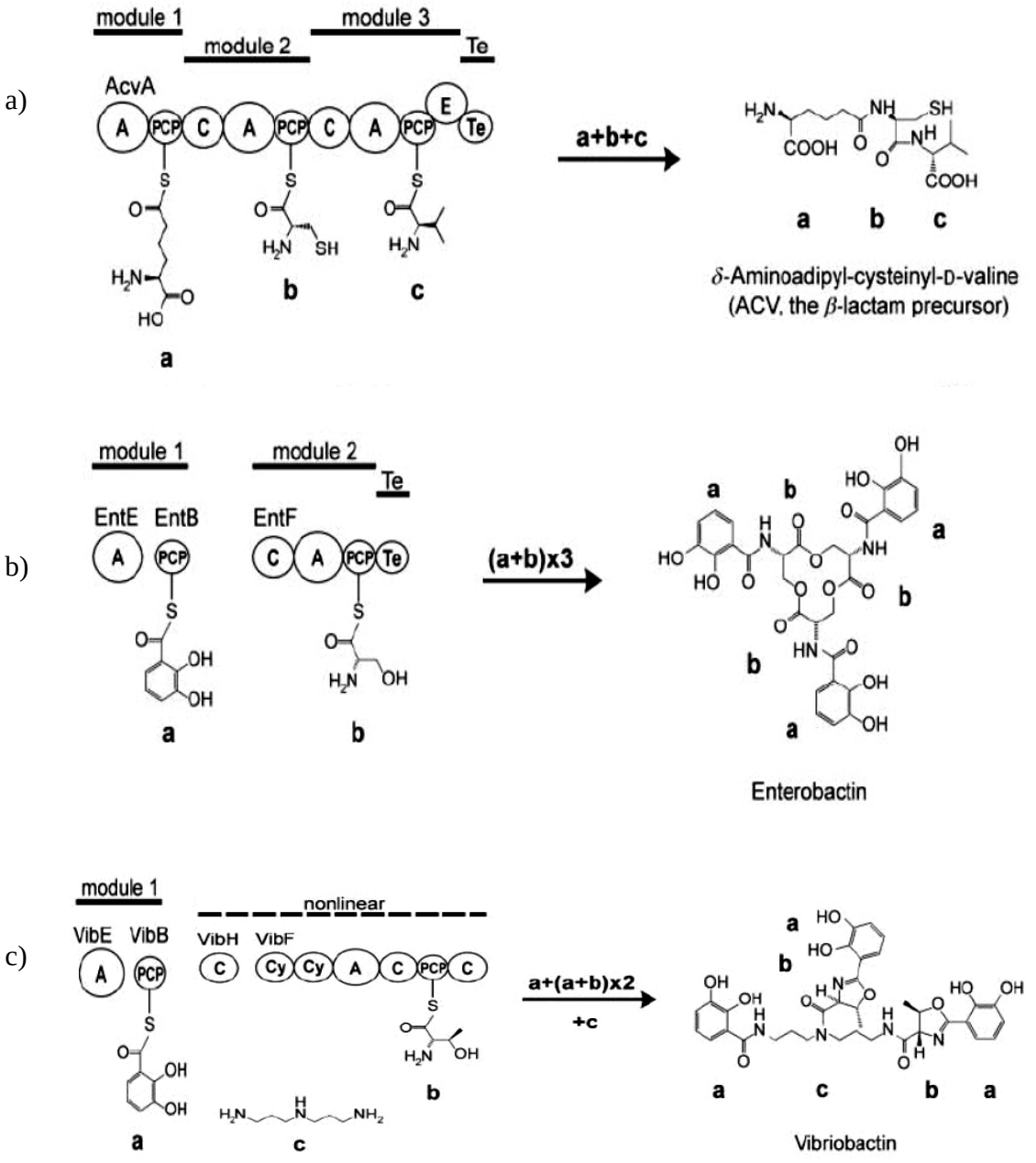


Figure 20. Les trois types de biosynthèse a) Biosynthèse linéaire : synthèse du tripeptide ACV, précurseur de la pénicilline et de la céphalosporine. b) Biosynthèse itérative : synthèse de l'entérobactine composée d'un dipeptide répété trois fois. c) Biosynthèse non linéaire : synthèse de la vibriobactine, peptide mixte NRPS/PKS

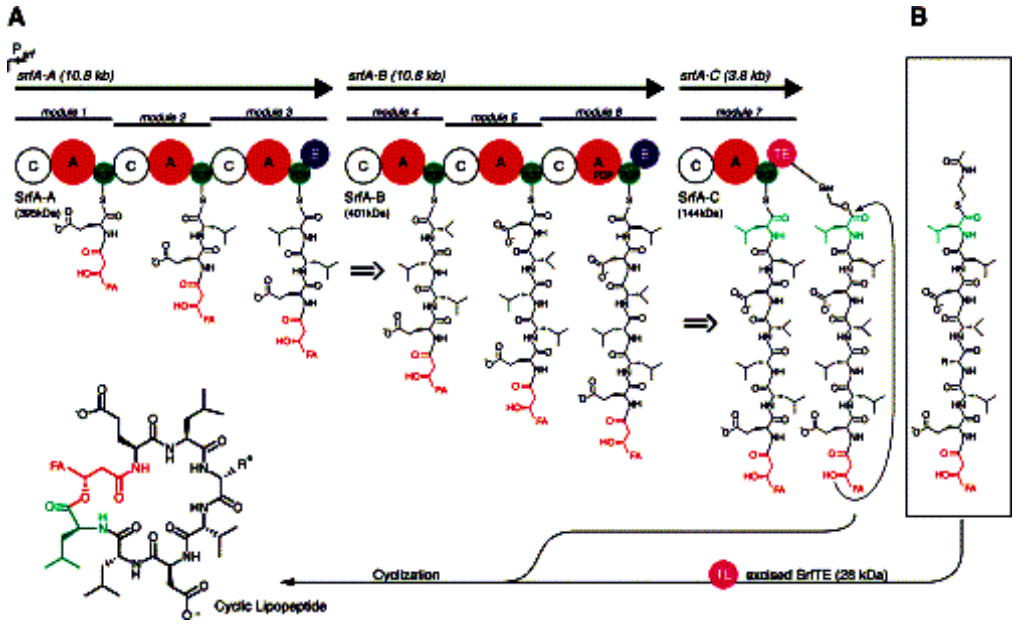


Figure 21. Surfactin Biosynthesis by the Modular Peptide Synthetase (A) The *srf* operon (top) with the three genes *srfA-A*, *srfA-B*, and *srfA-C* coding for the surfactin synthetase subunits shown below the genes. Bars indicate the positions of modules within the protein, whereas the individual domains are shown as colored balls: A, adenylation domain; PCP, peptidyl carrier protein domain; C, condensation domain; E, epimerization domain; TE, thioesterase domain. The 4-phosphopantetheinyl cofactors with their active thiol groups are shown with the corresponding peptides attached at their current synthesis states. The growing peptide chain is passed from left to right, until the linear product at the last PCP domain is cyclized to the lipopeptide by the TE domain. (B) The SNAC (S-N-acetyl cysteamine) acyl peptide mimic can be cyclized by the genetically excised SrfTE domain. The native peptide (R =DLeu) and the soluble substrate (R = DOrn) are illustrated. The β-hydroxy fatty acid (FA) is likely to be attached by the N-terminal C domain.

To date, the majority of characterized nonribosomal peptide synthetases and their nonribosomal peptide products have been from bacteria, especially spore-forming gram-positive soil bacteria such as *Bacillus* and *Streptomyces* species (Schwarzer & Marahiel, 2001; von Dohren *et al.*, 1997). Relatively fewer fungal genes encoding nonribosomal

peptide synthetases have been fully sequenced and their corresponding peptide structures determined. These include *Tolypocladium inflatum (niveum) simA*, which controls production of the immunosuppressive drug cyclosporine (Weber *et al.*, 1994), *Penicillium chrysogenum acvA*, which controls production of the antibiotic penicillin (Baldwin *et al.*, 1991), *Ustilago maydis sid2* and *Aspergillus nidulans sidC*, each of which controls production of a siderophore (Eisendle *et al.*, 2003; Yuan *et al.*, 2001), *Claviceps purpurea cpps1* and *cpps2*, both of which are involved in the synthesis of ergot alkaloids (Correia *et al.*, 2003; Tudzynski *et al.*, 1999), *Hypocrea virens tex1*, which controls peptaibol synthesis (Wiest *et al.*, 2002), *Leptosphaeria maculans SirP*, which controls sirodesmin biosynthesis (Eisendle *et al.*, 2003), and three genes that control production of peptide virulence effectors: *HTS1*, which controls production of HC toxin by *C. carbonum*, a maize pathogen (Scott-Craig *et al.*, 1992), *AMT1*, which controls production of AM toxin by *Alternaria alternata*, an apple tree pathogen (Johnson *et al.*, 2000), and *Esyn1*, which controls production of enniatin by *Fusarium scirpi* and other *Fusarium* spp., potato and tomato pathogens (Haese *et al.*, 1993; Herrmann *et al.*, 1996).

1.6 Hybrid between PKS and NRPS

The structural and catalytic similarities between NRPSs and PKSs support the idea of combining individual NRPS and PKS modules for combinatorial biosynthesis. Recent advances in cloning and characterization of biosynthetic gene clusters for naturally occurring hybrid polyketide-peptide metabolites have provided direct evidence for the existence of hybrid NRPS-PKS systems (Metz, *et al.*, 2001, Niermanm, *et al.*, 2005, Sebastian, *et al.*, 2007). Sequencing genome of several fungi including *A. fumigatus* and other filamentous fungi have revealed the presence of at least one gene that encodes a "hybrid" enzyme possessing domains typical of both PKS and NRPS. While such hybrid genes were described some time ago for bacteria (Du and Shen, 2001), only recently have the products of a few of the predicted fungal hybrid *PKS-NRPS* genes been determined. These include *fusA*, which is required for synthesis of a precursor of fusarin C in *Fusarium moniliforme* and *F. venenatum* (Song, *et al.*, 2004) a gene required for equisetin biosynthesis in *F. heterosporum* (Sims, *et al.*, 2005) and a gene that encode stenellin synthetase from *Beauveria bassiana* (Eley, *et al.*, 2007).

Hybrid peptide-polyketide natural products can be divided into two classes: (i) those whose biosyntheses do not involve functional interaction between NRPS and PKS modules; and (ii) those whose biosyntheses are catalyzed by hybrid NRPS-PKS systems involving direct interactions between NRPS and PKS modules (Du & Shen, 2001). It is the latter systems that are most likely amenable to combinatorial biosynthesis. The same catalytic sites appear to be conserved in both hybrid NRPS-PKS and normal NRPS or PKS systems, with the exception of the ketoacyl synthase domains in hybrid NRPS-PKS systems which are unique. KS domain has been considered the most conserved domain in normal PKS, while A domain is the most conserved in NRPSs. In some Hybrid systems in bacteria both the KS domain of PKSs and A domain of NRPS has been found (Zhu *et al.*, 2009). Specific linkers may play a critical role in communication, facilitating the transfer of the growing intermediates between the interacting NRPS and/or PKS modules. In addition, phosphopantetheinyl transferases with broad carrier protein specificity are essential for the production of functional hybrid NRPS-PKS megasynthetases.

In a previous study in bacteria, two patterns were used to identify KS domains belonging to hybrids between NRPS and PKS I and, more precisely, KS domains preceded by an NRPS, thus acting on a AA chain (Moffitt and Neilan 2003). Those two patterns were N(DE)KD at 22 AAs upstream from the cysteine active site in the KS domain and the VQTACSTS, which replaced the conserved pattern VDTACSSS of a typical KS domain (AA modification is shown with underline) (Jing *et al.*, 2008). In case of fungi, no such AAs modification has been ever reported in the literature.

1.7 Biosynthetic pathways of different fungal polyketide metabolites

1.7.1 Biosynthetic pathway of OTA

Up to this date, the biosynthetic pathway OTA is not yet fully established. A strictly theoretical consideration of the structure of the ochratoxin A molecule suggests that both an aromatic and an aliphatic pathway could be involved in its biosynthesis. However, certain initial experiments ^{13}C and ^{14}C labelled precursors feeding has shown that phenylalanine was incorporated into OTA, whereas ochratoxine α was constructed from

five acetae units with one carbon addition at C-7 from methionine (Searcy *et al.*, 1969; Steyn & Holzapfel, 1970). Phenylalanine portion of OTA molecule is synthesized via shikimic acid pathway, while the dihydroisocoumarine portion is synthezied via pentaketide pathway. Huff and Hamilton (1979) recognizing the lack of research in the pathway of OTA biosynthesis, proposed a hypothetical biosynthetic scheme, although this unfortunately ignored the ubiquitous OTB. The scheme proposed that mellein and hydroxymellein could probabily be the active precursors of OTA (Figure 22). Subsequently, the ochratoxine PKS was reported to produce mellein. Further it has been shown that there was no keto-enol tautomerism, but an unfunctionlised double-bond between C-6 and C-7 in the mellein pentaketide chain. The requisite methylation step was thought to occure after the production of mellein although the subsequent putative intermediates 7-methylmellein, 7-methoxymellein and 7-formylmellein have not been isolated from fungal fermentation producing ochratoxine (Abell *et al.*, 1983).

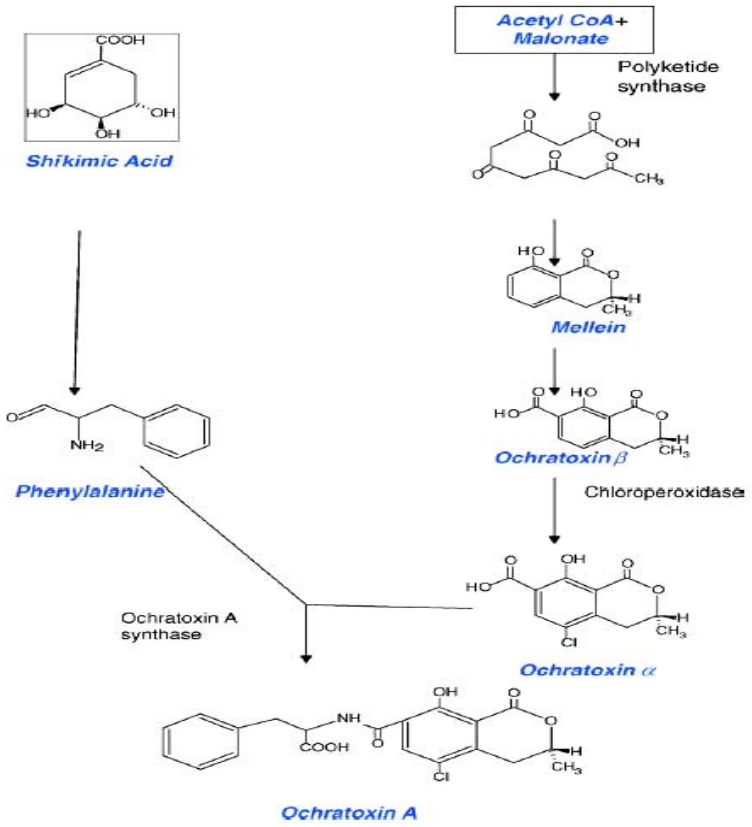


Figure 22. Hypothetical biosynthetic scheme of OTA (Huff and Hamilton, 1979)

However in some more recent experiments, incoroporation of labelled precursors of OTA to the growing cultures of OTA producing fungi *A. ochraceus*, decanted the hypothesis about the importance of mellein and hydroxymellein for OTA (Harris & Mantle, 2001). Relative incorporation of labelled putative intermediates ochtatoxin α and β and mellein indicated a strong preferential role of α, moderate role of β but found no role of mellein into OTA production.

The principal step during the biosynthesis of isocoumarine portion of OTA is the decarboxylative condensation of one acetate unit and 4 malonate units in the presence of polyketides synthase enzyme. The skeleton then undergoes cyclisation, aromatisation, methylation, oxydation and chlorination reactions (Steyn & Holzapfel, 1970). Once formed, the polyketide chain is then modified by the formation of a new lactone (synthesis of mellein) and by the addition of a carboxylic group (synthesis of ochratoxin ß). Later on, the chlorine atome is incorporated by the action of a chloroperoxidase (synthesis of OTA). Finally, the ochratoxine A synthase catalyses the chain elongation of OTA at phénylalanine.

The existence of multiple pathways (eromatic and aliphatic) for the biosynthesis of OTA has been supported by the presence of OTA *pks* gene in one producer strain and absence in the other. Recently it has been demonstrated that OTA *pks* genes are different between *A. ochraceus* (O'Callaghan *et al.*, 2003) and *P. nordicum* (Geisen *et al.*, 2004). Further, in *Penicillium*, Geisen *et al.*, (2004) has observed that *P. verrucosum* does not contain the same *pks* of *P. nordicum* which is important for the biosynthesis of OTA.

1.7.2 Biosynthetic pathway of aflatoxin B1

Aflatoxins, a group of highly reduced polyketide-derived furanocoumarins are the most toxic and carcinogenic compounds among the known mycotoxins produced by many species of *Aspergillus* and *Penicillium*. Among the at least 16 structurally related aflatoxins characterized, however, there are only four major aflatoxins, B1, B2, G1, and G2 (AFB1, AFG1, AFB2, and AFG2), that contaminate agricultural commodities and pose a potential risk to livestock and human health (Jiujiang *et al.*, 2004).

Aflatoxins are naturally occurring mycotoxins that are. The biosynthetic pathway of this mycotoxin has been extensively studied and is used as a model for understanding biosynthetic schemes of various fungal polyketides. It has been described that several additional steps are included in the modified version of biosynthetic scheme of aflatoxin B1 (Anderson, 1992). These additional steps in the pathway giving the sequence: acetyl CoA + malonyl CoA → polyketide → norsolorinic acid → averantin → averantinin → averufin → hydroxyversicolorone → versiconal acetate → versiconal → versicolorin B (VB) → versicolorin A (VA) → demethylsterigmatocystin (DHST) → sterigmatocystin (ST) → O-methylsterigmatocystin (OMST) → aflatoxin B1 (AFB1). The structures of the intermediates between versiconal acetate and AFB1 are shown in Figure 23.

Cell-free preparations that catalyse conversion in the portions of the pathway aceW1 plus malonyl CoA to norsolorinic acid, averufin to versiconal acetate, VA to ST, and the formation of AFG1 and AFG2 have not been reported, apparently because of the instability of these enzymes (Anderson, 1992).

The dehydrogenase that catalyses the conversion of norsolorinic acid to averantin has been purified to near homogeneity by affinity chromatography on a norsolorinic acid affinity column (Chuturgoon *et al.,* 1990). A cell-free system converted averantin to averufin (Yabe *et al.,* 1991). Versiconal acetate was hydrolysed in cell-free systems to versiconal and VB (Anderson & Chung, 1990; Hsieh *et al.,* 1989). The enzyme that catalyses the conversion of versiconal to versiconal cyclase (VC) has been purified to homogeneity. VB has the same configuration of the dihydrofuranofuran moiety as the aflatoxins. VC, the form isolated from cultures, is a racemic mixture of VB and its enantiomer. The optical form derived from versiconal acetate was not determined in these studies. However, McQuire *et aI.* (1989) reported that VB was the product of the cell-free hydrolysis and subsequent cyclization of versiconal acetate (McQuire *et al.,* 1989).

The hemiacetal of VA was reduced to VB by NADPH in cell-flee extracts (Anderson & Chung, 1990; McQuire *et al.,* 1989). No versiconal was formed. The absence of versiconal may be due to the versiconal cyclase activity described above, which would

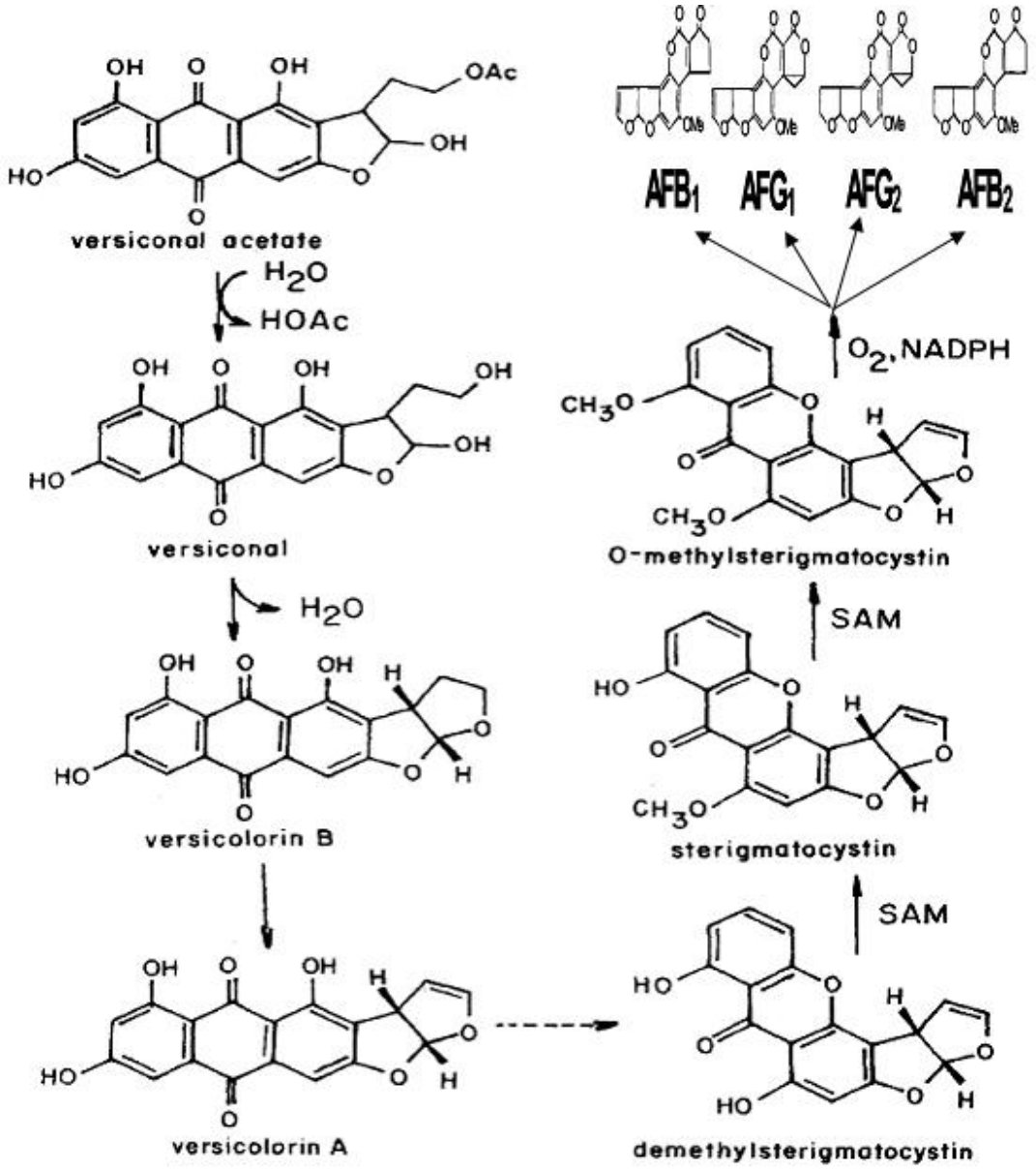


Figure 23. Aflatoxin biosynthetic pathway from versiconal acetate to aflatoxin B1, B2, G1 and G2 in *Aspergillus sp*. (Anderson, 1992; Jiujiang *et al.*, 2004) Solid arrows indicate reactions observed in cell-free systems. Dashed arrows represent reactions that have not been reported to occur in cell-free systems. SAM = S-adenosylmethionine.

convert versiconal to VB. The hemiacetals AFB2a and AFG2a were also reduced, but to the open chain derivatives of AFB2 and AFG2 (Anderson, 1992). The cell-free extracts therefore lacked cyclase activity for the latter compounds. It is doubtful that these reactions participate significantly in the pathway of aflatoxin biosynthesis.

VC [9-^{13}C] (racemic mixture, 50% VB) was converted to AFB1 and AFB 2 in cultures of *Aspergillus parasiticus* (McQuire *et al.* I989). VB was converted to VA in a cell free system (Yabe ef *al,* 1991). These observations support the positioning of VB at a branch point to AFB2 via VA and ST (Figure 23) or to AFB2 via DHST. DemethylST was converted to ST by a ceil-free system plus S-adenosylmethionine (Figure 23). DHdemethylST was similarly converted to DHST.

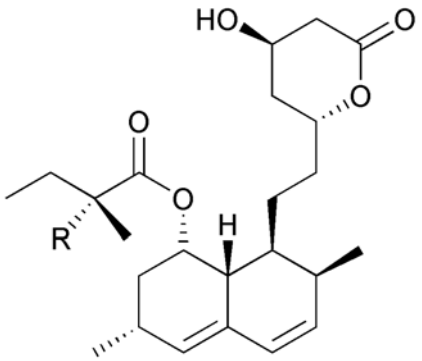
The cell-free conversion of ST to AFB1, first reported by Singh & Hsieh (I976), has been found to involve two enzymes: a soluble transmethylase that catalyses the O-methylation of ST by S-adenosylmethionine, and an oxygenase in the particulate cell fraction that catalyses the subsequent oxidation of OMST to AFB 1 (Anderson, 1992) (Figure 23). The oxygenase also catalysed the conversion of DHOMST to AFB1. The transmethylase has been purified to homogeneity (Bhatnagar *et al.*, 1988), and monoclonal antibodies to the enzyme have been prepared (Bhatnagar *et al.*, 1989).

1.7.3 Biosynthetic pathway of Lovastatin

The highly reduced type polyketide “Lovastatin” (Figure 24)(also known as mevinolin, monacolin K, and Mevacor) is a potent cholesterol-lowering polyketide metabolite (Alberts *et al.*, 1980) and a precursor for synthesis of the widely-prescribed drug like simvastatin (W. F. Hoffman *et al.*, 1986).

Biosynthetic studies on lovastatin (Figure 25) with fungi, such as *Aspergillus terreus*, show that its assembly *via* a polyketide pathway involves initial construction of dihydromonacolin L **5** (John *et al.*, 2002). The cooperation of polyketide synthas (PKSs) encoded by the *lovB* and *lovC* genes accomplish 35 steps necessary to generate **5** (Figure 25) (J. Kennedy *et al.*, 1999) . In the absence of the LovC protein, which imparts enoyl

reductase activity to the complex, the LovB iterative type I PKS enzyme generates truncated pyranones **6** and **7.** During normal biosynthesis, oxidative transformations of



1 R = H, Lovastatin
2 R = CH_3, Simvastatin

Figure 24. Chimical structure of lovastatin and simvastatin

the PKS product **5** introduce a second double bond in the decalin (decahydronaphthalene) system andadd the C-8 hydroxyl to form monacolin J **9.** The 2-methylbutyryl side chain is produced by a separate PKS encoded by *lovF*, and further used by LovD to directly acylate **9** and yield **1.** The function of LovA, a protein essential for the formation of **1**, is not fully understood, but it has sequence homology to P450 enzymes and is probably involved in the oxidative conversion of **5** to introduce the diene system present in **9**. Biosynthetic studies on compactin using the fungus *P. aurantiogriseum* indicate a labelling pattern analogous to that seen in lovastatin**,** but the genetic machinery required for the formation of compactin has not yet been reported.

Further, biosynthetic studies with fungi (e.g., *A. terreus*) show that lovastatin (1) is a polyketide (Figure 24) derived from 11 acetate units, molecular oxygen, and two methyl groups from S-adenosyl-l-methionine (SAM)(Auclair *et al.*, 2001). The polyketide synthase (PKS) steps of the biosynthesis of lovastatin and compactin involve the head-to-tail assembly of all the acetate molecules as well as a cyclization to afford 4a,5-dihydromonacolin L and its 6-desmethyl analogue, respectively (Auclair *et al.*, 2001). Compounds 4a,5-dihydromonacolin L and 6-desmethyl are further transformed into

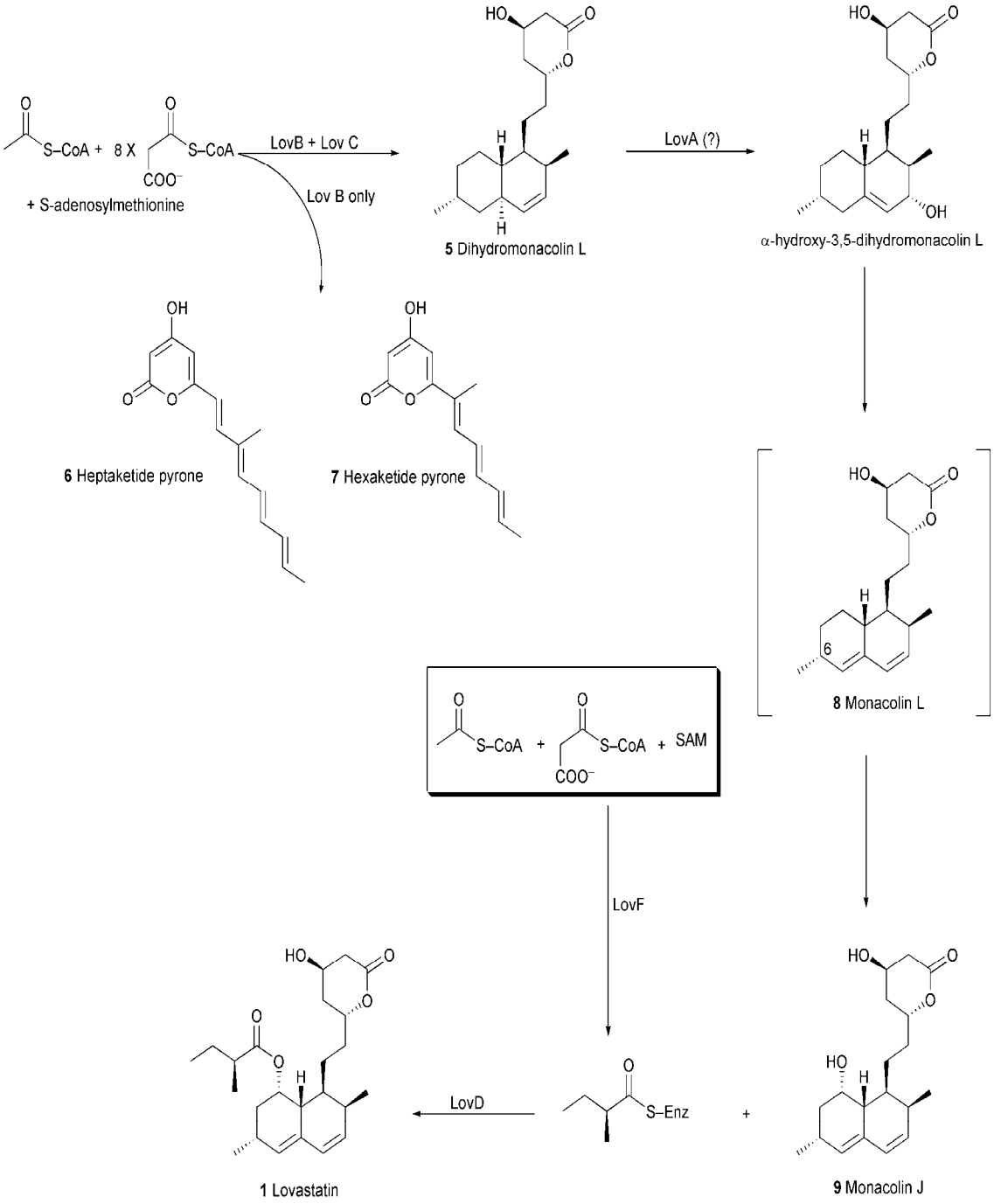


Figure 25. Biosynthetic scheme of Lovastatin (J. Kennedy *et al.*, 1999).

lovastatin and compactin, respectively, through post-PKS modification steps that may proceed as proposed by Endo and co-workers (Figure 25). Using cell-free extracts of *M. ruber*, they demonstrated the conversion of 4a,5-dihydromonacolin L into 3a,hydroxy-3,5-dihydromonacolin L, which is suggested to spontaneously dehydrate to give 6. Hydroxylation at C-8 of monacolin L (6) by molecular oxygen present in the cellfree system of Endo and co-workers provides monacolin J (7), but researchers at Merck concluded that 6 is formed from 5 only as an artifact of isolation rather than as a discrete product of biosynthesis since no monacolin L (6) can be detected in freshly harvested *A. terreus* cultures, whereas an increasing amount appears during the isolation of lovastatin (1). The nonaketide 7 is finally converted to lovastatin (1) in the cell-free extract by esterification with the diketide (2R)-methylbutyric acid (8).

1.7.4 Biosynthetic pathway of Patulin

Patulin is a potent antibiotic and mycotoxin which is synthesized from acetyl-coenzyme A by several *Penicillium* species (Clegler *et al.*, 1971). Study of the biosynthesis of patulin is important due to many reasons. First, it is important to understand the hypothesis of Birch who stated that acetate is the basic unit in the biosynthesis of many polyketide secondary metabolites, based on its work highlighting the incorporation of radiolabelled acetate in 6-methylsalicylic acid (6-MSA) (Birch et al; 1955). This work has confirmed the existence of a new class of previously suggested natural molecules poyletides. This discovery was important because it provided the first evidence that eukaryotic cells could synthesize aromatic rings from acetate and not from sugars derived from the shikimic acid pathway. Secondly, the first enzyme in the biosynthetic pathway of patulin i.e 6-MSA, was the first PKS studied *in vitro* (Lynen et Tada, 1961). Finally, studies on the production of patulin in submerged cultures of *P. urticae* have provided evidence that secondary metabolism is a phenomenon associated with the stationary phase of the fungi (Puel, 2007a).

The patulin biosynthetic pathway is well characterized, and the route from acetyl-coenzyme A to gentisaldehyde has been firmly established via 6-methylsalicylic acid, m-

cresol, and m-hydroxybenzyl alcohol (Figure 26) (Forrester & Gaucher, 1972; Zamir, 1980).

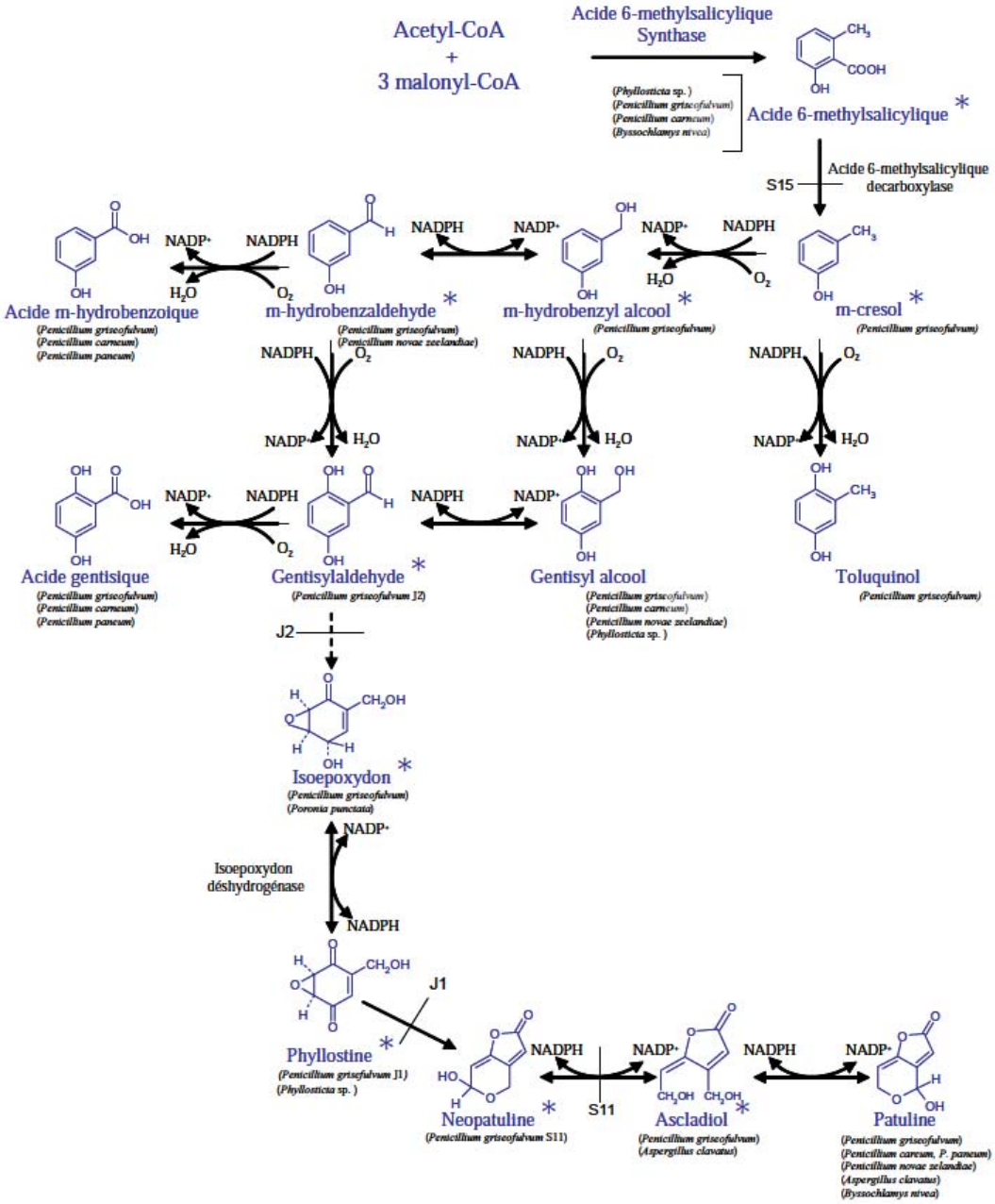


Figure 26. Biosynthetic pathway of patulin and related compounds. Compounds with an asterisk are proven precursors of patulin (Forrester & Gaucher, 1972; Zamir, 1980).

Until 1978 the post-gentisaldehyde portion of the pathway was believed to consist of a single, dioxygenase-mediated ring cleavage. Since then extensive studies of five pat ulin-minus mutants have shown that patulin is synthesized via isoepoxydon, phyllostine, and neopatulin (isopatulin) (Gaucher, 1979; Sekiguchi & Gaucher, 1977).

The first step in the production of patulin resids in the formation of 6-methylsalicylic acid, by the condensation of one molecule of acetyl-coA and 3 molecules of malonyl-coA due to the actions of a single multi-functional enzyme, 6- methylsalicylic acid synthase (6-MSAS). It has been shown that 6-MSA specifically marked has converted to patulin (Bassett & Tanenbaum, 1958). The number of enzymatic steps permitting the transformation of 6MSA to patulin is estimated to about 10. 6-MSA is first decarboxylated to m-cresol, the methyl group is then oxidized to form an aldehyde group, the aromatic core then hydroxylated to achieve gentisylaldehyd.

From gentisaldehyde, it appears that the succession of different precursors is much more blurred. In fact, the transfer of gentisaldehyde into a bicyclic structure like patulin needs the opening of the ring through which can either be mediated by a monooxygenase or dioxygenase like that occur in the bacterial world.

Isolation and characterization of P3, S15, J2, J1 and S11 mutants unable to produce patulin has helped to clarify some of the doubtful site of patuline biosynthesis (Sekigouchi *et al.*, 1983). The study of Sekigouchi *et al* (1983) facilitated the isolation of four other potential precursors of patulin , the (+)-isoepoxydon and (-)-phyllostine (2-hydroxymethyl-5, 6 epoxy-1,4 benzoquinone), the néopatuline and ascladiol (Puel, 2007a). All these precursors have been found to be converted *in vivo* or *in vitro* to patulin.

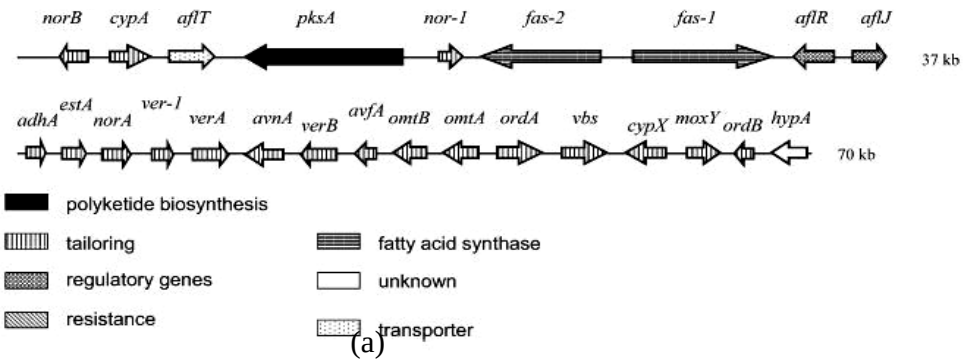
1.8 Organization of polyketide gene clusters

The adaptation of an organism to environmental conditions requires the cooperation of several genes that contribute to its survival. In a metabolic chain, the product of an enzymatic reaction must be taken very quickly by following the enzyme to ensure a correct speed of formation of the final product and avoid being destroyed by the possible

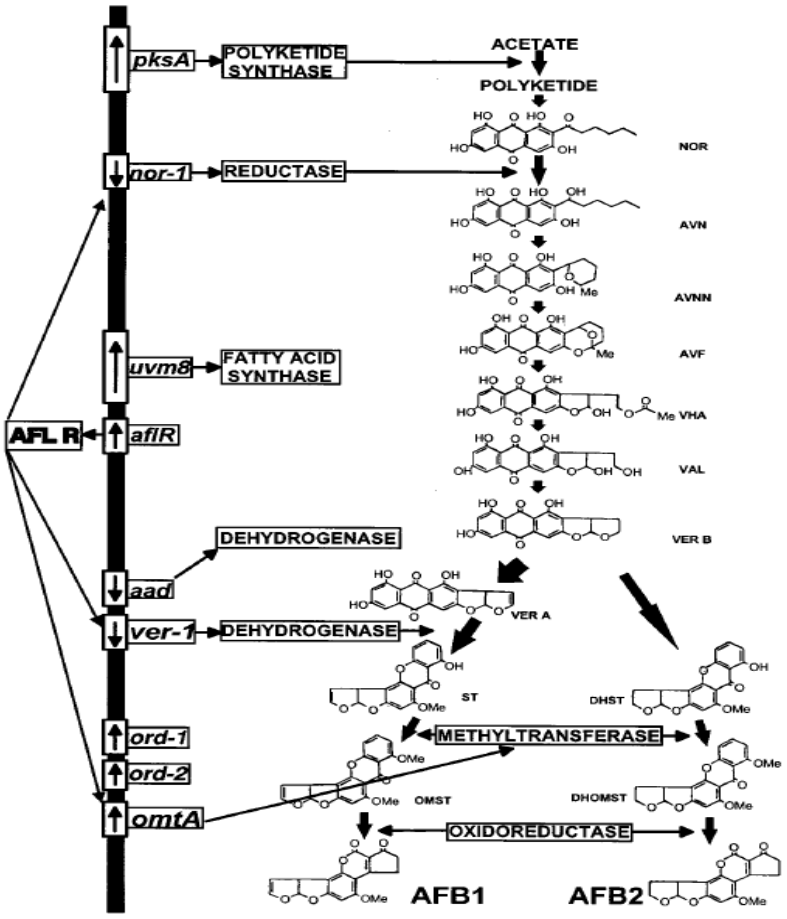
reactions. So an enzymatic assembly in the form of complex or enzymatic cluster (Sidhu, 2002; Yu & Keller, 2005) is therefore a necessity. If the genes are highly related, they can form units or clusters. The genes catalyzing secondary metabolites are generally organized in such clusters. Several polyketide clusters are identified in bacteria and in fungi.

In fungi, several clusters coding for polyketides have been studied. These clusters include clusters for the biosynthesis of mycotoxins and phytotoxins (aflatoxines, fumonisine, trichothecene, gibberelline, ergot alkaloid, paxilline), antibiotics (cephalosporine and penicilline), melanins and some pharmaceutical products (lovastatine et compactine). The production of these metabolites are under control of the clusters that include all the genes (or nearly all) of the biosynthetic pathway of the molecule. It has been identified that during the biosynthesis of aflatoxins more then 27 enzymes and regulatory factors encoded by a cluster group of genes are required. In *A. flavus*, at least 25 genes responsible for the production of this toxin have been identified. These genes are grouped in a cluster of 70 kb (Figure 27a), which is composed of three major genes: two fatty acid synthases subunits (*Fas1* 5,8 kb and *Fas2* 5,1 kb), a PKS of 6.6 kb (*pksA*) and 22 other genes of about 2 kb each (Bhatnagar *et al.*, 2003; Yu *et al.*, 2004a). Some species of *Aspergillus*, for example *A. nidulans*, produce precursor of aflatoxin i.e. sterigmatocystin, in which the genes are grouped in a cluster of 60 kb that has genes in common with those of aflatoxin, but not in the same order.

Different genes and their corresponding enzymes involved in the biosynthesis of aflatoxin A and B have shown in figure 27b. Four of the 25 aflatoxines producing enzymes have found to be encoded by *verA*, *ver-1*, *aflX* and *aflY* (Cary et al., 2006). These 4 enzymes are required for conversion of versicolorin A (VA), to demethylsterigmatocystin. Studies have shown that in the two clusters, aflatoxins and sterigmacystine, the *aflR* gene acts as a regulator. It codes for a specific protein of zinc-binding DNA type, required to activate the transcription of most genes structure. The transcription of the biosynthetic pathway can be activated when the protein AflR fix itself to palindromic sequence 5'-TCGN_5CGA-3' of the promoter region structural gene as



(a)



(b)

Figure 27. Gene cluster of aflatoxin in *Aspergillus spp.* (a) organization of genes, (b) Genes and their corresponding enzymes in the aflatoxin B1 and B2 biosynthetic pathway (Schümann & Hertweck, 2006; YU *et al.*, 1995).

described in *A. nidulans*, *A. flavus* and *A. parasiticus* (Jin & Dong-K, 2007). In *A. sojae*, a non aflatoxines producing strain utilised in food industry, the *aflR* gene is inactive.

Biosynthetic gene cluster of another fungal polyketide cytochalasan in *P. expensum* (Julia & christian, 2007) is composed of several genes among which 7 are identified and characterized (Figure 28). The flanking region of this cluster encodes a hybrid PKS-NRPS (CheA), an enoyl reductase (CheB) and three putative oxygenases, CheD, CheE, and CheG. Chaetoglobosin biosynthesis is likely regulated by the two putative C6 transcription factors CheC and CheF.



Figure 28. Organization of the cytochalasan biosynthesis gene cluster in *P. expensum*. Deduced functions of gene products: CheA, PKS-NRPS; CheB, enoyl reductase; CheC, CheF, regulators; CheD, CheE, CheG, oxygenases.

There are several other examples in which genes encoding a polyketide are grouped in the form of clusters on the genome of that particular organism. For example, in case of fumonisin biosynthesis, 15 genes are regrouped in a cluster of 75 kb (Figure 29a) in *Gibberella moniliformis* (Robert *et al.*, 2003). Initially a cluster of five fumonisin biosynthetic genes, FUM1, FUM6, FUM7, FUM8, and FUM9 was reported (Seo *et al.*, 2001). More recently 10 additional genes located immediately downstream of FUM9 has been identified (figure 29a) (Robert et al., 2003). This conclusion is based on the observation that the 10 newly identified genes are coregulated with the five previously identified FUM genes and that expression of all 15 genes in the cluster is correlated with fumonisin production. The predicted functions of most of the 10 new FUM genes are consistent with activities expected to be required for fumonisin biosynthesis or self-protection. Interestingly, none of the 15 genes within the cluster appears to be a regulatory gene. *FUM1* has been identified to be an active PKS, which was predicted to

catalyze the synthesis of a linear 18-carbon polyketide that forms C-3–C-20 of the fumonisin backbone.

The lovastatin biosynthesis genes are grouped in a cluster of 37 kb contain about 17 genes with two active PKS (*lovA* and *lovB*) and compactin biosynthesis genes are grouped in a cluster of 38 kb containing 9 genes with two active PKSs (*mlcA* and *mlcB)* (Figure 29b).

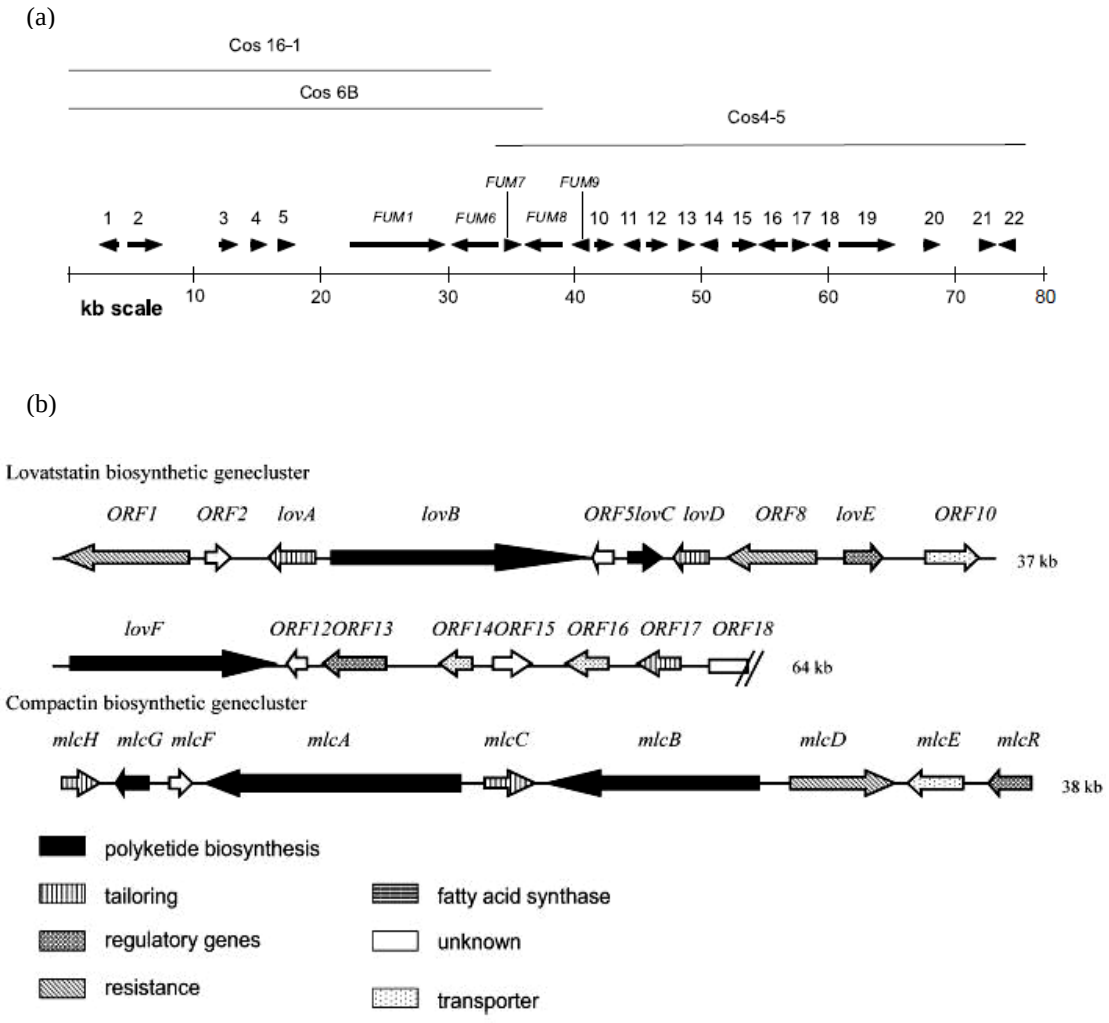


Figure 29. Organization of the gene cluster for fungal PKS of a) Fumonisin: Map of cosmid clones Cos16-1, Cos6B, and Cos4-5 showing positions of newly identified ORFs and previously identified genes FUM1 and FUM6–FUM9. Arrows indicate locations and orientations of genes and b) lovastatine and compactine (Schümann & Hertweck, 2006)

The cluster organization is not necessary for all secondary metabolites. For example in case of melanin biosynthesis, the biosynthetic genes are in cluster in *A. alternata* but not in *C. lagenarium* (Keller & Hohn, 1997). Walton (2000) has suggested that this organisation permit to regulate and coordinate different steps of a biosynthetic pathway. It has also been proposed that the organisation of genes in a cluster assure their resistance and stability during the course of evolution (Geisen, 1996; Geisen *et al.*, 2004). There equally exist the possibility that a gene away from the cluster participle with genes of the cluster in the same biosynthetic pathway. It is the case of *ZFR1* gene which is a fumonisin regulation gene but which is not part of the 45kb gene cluster.

Hence study of the different polyketide biosynthesis genes concludes that;

- The biosynthesis of polyketides is a very complex process involving several type of enzymes systems other than PKS and PKS-NRPS.
- In most cases genes involved in the biosynthesis of a polyketide is grouped in the form of cluster.
- They are cases where polyketide genes are not grouped in cluster.
- In some particular cases some genes specilly regulatory genes can exist away from the particular polyketide genes cluster.

Materials and methods

2- Materials and Methods

2.1 Materials

2.1.1 Liste of kits utilized

Kit	Supplier
FastDNA SPIN kit	MP Biomedicals & Qbiogene, Illkirch, France
QuantiTect™ SYBR® Green PCR Kit	Qiagen, Courtaboeuf, France
Colonnes d'immuno-affinité OchraPrep	r-Biopharm, St Didier Au Mont D'Or, France
EZNA Fungal DNA Miniprep	Biofidal, Vaulx en Velin, France
RapidPURE™ Plasmid Mini Kit	MP Biomedicals & Qbiogene, Illkirch, France
JETQUICK PCR Spin Kit 50	MP Biomedicals & Qbiogene, Illkirch, France
JETQUICK Gel Extraction Spin 50	MP Biomedicals & Qbiogene, Illkirch, France
Tri Reagent	Euromedex, Paris, France
Advantage, Clontech	BD Biosciences, Le Pont de Claix, France
TOPO TA Cloning	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
PCR-Select cDNA subtraction kit	Clontech Laboratories France
SuperCos 1 Cosmid vector kit	Stratagene USA and Canada
Gigapack III Gold packaging extract	Stratagene USA and Canada
Expand Long templqte PCR system	Roch chemical Germany

2.1.2 List of products utilised

- Agarose Mol Bio Grade (Promega, Charbonnières, France)
- Agar (Difco, Fisher Bioblock Scientific, Illkirch, France)
- Ammonium Sulfate (Fluka, Saint Quentin Fallavier, France)
- Ammonium nitrate (Fluka, Saint Quentin Fallavier, France)
- Ammonium chlorid (Sigma Aldrich, Saint Que ntin Fallavier, France)
- Ammonium molybdat (Fluka, Saint Quentin Fallavier, France)
- Acetonitrile for CLHP (Fisher Bioblock Scientific, Illkirch, France)
- Acetic acide (Sigma Aldrich, Saint Quentin Fallavier, France)
- Ampicilline (Sigma Aldrich, Saint Quentin Fallavier, France)
- CTAB (Sigma Aldrich, Saint Quentin Fallavier, France)
- Catechin (Sigma Aldrich, Saint Quentin Fallavier, France)
- Chloroform (Fisher Bioblock Scientific, Illkirch, France)
- Coper sulfate (Fluka, Saint Quentin Fallavier, France)
- Diethyl Pyrocarbonate (Sigma Aldrich, Saint Quentin Fallavier, France)
- DL- malic acid 99 % (Fisher Bioblock Scientific, Illkirch, France)
- D(+)-Glucose anhydre (Fluka, Saint Quentin Fallavier, France)
- Ethanol (Fluka, Saint Quentin Fallavier, France)
- EDTA (Sigma Aldrich, Saint Quentin Fallavier, France)
- Ethidium bromid (Sigma Aldrich, Saint Quentin Fallavier, France)
- Fructose (Fluka, Saint Quentin Fallavier, France)
- Glycerol (Fluka, Saint Quentin Fallavier, France)
- Hygromycine B (Calbiochem, VWR International, Fontenay sous Bois, France)
- Hydrochloric acide (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 enzyme (Sigma Aldrich, Saint Quentin Fallavier, France)
- Manganese sulfate (Fluka, Saint Quentin Fallavier, France)

- Malt Extrait (Difco, Fisher Bioblock Scientific, Illkirch, France)
- Magnesium sulfate heptahydrat (Fluka, Saint Quentin Fallavier, France)
- Methanol for CLHP (Fisher Bioblock Scientific, Illkirch, France)
- Maltose (Fluka, Saint Quentin Fallavier, France)
- Peptone (Fisher Bioblock Scientific, Illkirch, France)
- PDA medium (Potato Dextrose Agar) (Difco, Fisher Bioblock Scientific, Illkirch, France)
- Potassium phosphate monohydrogen (Fluka, Saint Quentin Fallavier, France)
- Potassium phosphate dihydrogen (Fluka, Saint Quentin Fallavier, France)
- Potassium chlorid (Sigma Aldrich, Saint Quentin Fallavier, France)
- Potassium hydroxyd (Sigma Aldrich, Saint Quentin Fallavier, France)
- Polyethylene glycol 6000 (Sigma Aldrich, Saint Quentin Fallavier, France)
- Phenol -chloroforme- isoamyl alcohol : 25/24/1 (v/v/v) (MP Biomedicals & Qbiogene,Illkirch, France)
- Restriction enzymes: Ecor1, Not1, Sal1, Sma1. (MP Biomedicals & Q-biogene, I llkirch, France)
- Sodium nitrate (Fluka, Saint Quentin Fallavier, France)
- Sodium chlorid (Sigma Aldrich, Saint Quentin Fallavier, France)
- Sodium hydroxyd (Fluka, Saint Quentin Fallavier, France)
- Saccharose (Fluka, Saint Quentin Fallavier, France)
- Sodium Borate (Fluka, Saint Quentin Fallavier, France)
- Sodium sulfit (Na2SO3) (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)
- Tartric acid (Sigma Aldrich, Saint Quentin Fallavier, France)
- Tris (Sigma Aldrich, Saint Quentin Fallavier, France)
- Tris (Euromedex, Paris, France)
- Tris-Acetate, EDTA 50X (MP Biomedicals & Qbiogene, Illkirch, France)
- X-Gal (Euromedex, Paris, France)
- Yeast extrait (Difco, Fisher Bioblock Scientific, Illkirch, France)

- Zinc sulfate (Fluka, Saint Quentin Fallavier, France)

2.1.3 Apparatus used

- pH-meter (Corning- EEL modèle 109)
- High performance liquid chromatography (HPLC) (BIO-TEK, Milan, Italie) with a column C18 and manipulating software (PC) (Kroma 3000)
- 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)
- Microcentrifuge (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)
- Homogeniser (Ultra Thurax)
- UV-visible spectrophotometer (Philips PU 8600)
- Electrophoresis tank, horizontal system (Embi Tec, San Diego, CA)
- Vacuum system Visiprep of Supelco (Sigma Aldrich, Saint Quentin Fallavier, France)
- Thermocycler (Robocycler gradient 96 stratagene)
- Real time PCR (ABI PRISMR 7700; Applied Biosystems, Foster City, CA, USA).
- Table-top UV transilluminator
- Quantity one analysis software (BioRad France)
- Fast prep (Thermo electron corporation France)

2.1.4 Culture medium

a. 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)
$NaNO_3$……. 20 g
KCl…….. 5 g
$MgSO_4.7\ H_2O$…….. 5 g
$FeSO_4.7\ H_2O$……. 0,1 g
Distilled water …… qsp 500 mL

Solution C (500 ml)
K_2HPO_4……... 10 g
Distilled water …… qsp 500 mL

Solution Cu+Zn (100 mL)
$ZnSO_4$, $7H_2O$…… 1 g
$CuSO_4$, $5H_2O$…….. 0.5 g
Distilled water …… qsp 100 mL

b. Czapek Dox Agar (CZ)

The composition of CZ medium is same as CYA but without yeast extract incorporation.

c. Synthetic Medium (SM)

Preparation of MS medium needs advance preparation of solutions A and B. Composition of these solutions is as follow:

Solution A

Composition for 1 liter

NH_4NO_3 …….. 3 g

K_2HPO_4……26 g

KCl…….....……...1 g

Mg SO_4, 7 H_2O…….. 1 g

Mineral solution……..… 10 mL

Agar ……. …15 g

pH is adjusted to 6.5 with 6 N HCl

Mineral solution

Composition for 1 liter

$Na_2B_4O_7$. H_2O…….70 mg

$(NH_4)_6$ Mo_7O_{24}. $4H_2O$…….....….50 mg

$FeSO_4.7H_2O$ ……..1000 mg

$CuSO_4.5H_2O$ ……..…30 mg

$MnSO_4.H_2O$ …….....11 mg

$ZnSO_4.7H_2O$……1760 mg

pH is adjusted to 2 with 6 N HCl

Solution B

Glucose ……100 g/500 mL

Solution A and B are sterilized separately at 121 °C for 20 min. Final preparation of MS medium consists of a mix of 75 mL of solution A and 25 mL of solution B in an Erlenmeyer flask of 250 mL.

d. Malt Extract Agar (MEA)

Composition for 1 liter

Malt extract……. 20 g

Glucose…….....20 g

Peptone…….....20 g

Agar……. 15 g

Distilled water..……. qsp : 1L

Autoclave for 15 min at 121°C

e. 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

f. LB Broth

Composition for 1 liter

NaCl	……	10 g
Tryptone	……	10 g
Yeast extract	……	5 g

Adjust a pH 7.0 with 5 N NaOH

Add deionized H_2O to adjust final volume to 1 liter

Autoclave for 15 min at 121°C

2.1.5 Oligonucleotide primers used

Primer name	Sequence (5´-3´)
ATR1	YTG5GC5CCYTG5CC5GTDAA
ATR2	CATRTGRTGIGARTGRTAIGC
LCS1	CGGCCGCAATTCTTCGAA
LCS2	GCCATGCACAGCCACGCG
Aoat6-R	CGTCCTGGAAGACAAGGTGT
AoLc35-6disF	AAGCTCTAGAATCCGGTGGCCTG
AoLC6*hph*R	<ATCCCCGGGTACCGAGCTCGAATTC> < GGCTCGACGAACTGAACC>
AoLC6*hph*F	<TCGACCTGCAGGCATGCAAGCTGGC> <AACAGCTTTGGATTCGGT>
*hph*1F	GAATTCGAGCTCGGTACCCGGGGAT
*hph*1R	GCCAGCTTGCATGCCTGCAGGTCGA
Aoat6-F	ACGTGGTCGCTTAAAGAGGA
*hph*2F	CGGGGGCAATGAGATATGAAAAAG
*hph*2R	GAACCCGCTCGTCTGGCTAAG
TubF	CTCGAGCGTATGAACGTCTAC
TubR	AAACCCTGGAGGCAGTCGC
AoLC35-2R	CTGGAGGATCTCGCTGATGT
AoLC35-2L	TTTGATCGACCATTGTGTGC
AoKS1	CGGAAGGCCGGCCTAGATCCAGCC
AoKS1F	GAAGCCGTCGAGGCCGCCGGTCTG
AoKS1R	CAATGCGAATTGCCTCTATTTC
AoKSF	CGGCCAATCTGGGAGATTTGGC
LC12F	CTATGACTTACGCGGGACAAG
LC12R	AAGGCAGATACAATGGCCTGC
AT	YTG5GC5CCYTG5CC5GTDAA
ATR	CATRTGRTGIGARTGRTAIGC
LCF1	CTGATTCGTAGAATTTAC
LCF2	CTTCGGAATATCCGCCCA
LCF3	CTTCAGGTCAAGGGCGAG
5'lc12F	GCGATGCTCTTCGGGATG
3'lc12R	TTC AAA CGA GCT AAC AGT
5'*hph*R	<GTGCTCCTTCAATATCATCTTCTGT> <ACTGCGAAGTGGAATGCGACTTGTG>
3'*hph*F	<ATGCAGGAGTCGCATAAGGGAGAGC> <GGCATTTGATGAGGCTGGTTTGGAC>
LC12fullF	CAGATGGACCCGCAGCAG

LC12fullR	CATATTGACAACTGGCTG
SotLCF	TCTCCGCACCGCCATCAA
Aolc12F	AGAACGTGTATCATGCGCTCG
Aolc12R	GCATCGCGAAGTGGCTTGA
LC3 (Bingle *et al.*, 1999)	GCIGA(A/G)CA(A/G)ATGGA(T/C)CCICA
LC5c (Bingle *et al.*, 1999)	GTIGAIGTIGC(G/A)TGIGC(T/C)TC
MS1	GCCGCCGGCGTTGACCCGATGAC
MS2	CGGCCIAAGGIAA(T/C)ICCITG
MS3	CTTAGCAAATCATTCACCATGGAC
MS4	(C/T)T(G/C)(A/G)(T/C)(C/T)(A/T)TCCAGCACGCCIGC
MS5	ATCCAAACGCTAGACCAACTCGGC
MS6	CATTTTCGATCGCCTGCCATGCC
MSdF1	ATGCCTTTCTTGGATCCGTCCTCG
MSdR1	CTAGGCAGCTAGTTTCTCCGCGAAC
MSdF	CCGATCGTGTTCAGATCTTGACCTTCCTGATG
MSdR	ACTAAGTTGGGGATGTCCTGAGATGACGAGA
3'MsR :	CGGGGGCAATGAGATATGAAAAAG
3'MsF :	GAACCCGCTCGTCTGGCTAAG

2.2 Methodology

2.2.1 Inoculums preparation and conservation

A spores suspension (10^6spores / mL) of *A. westerdijkiae* was spread on a petri plate containing CYA medium. After 10 days of incubation at 25°C, 5 to 10 mL of 0.01 % Tween 80 solution was spread on each plate. The spores were scratched with a sterile blade and filtered with a gaze. Counting of the spores was performed through Thoma Bright line counting chamber.

Fungal strain was conserved at -20 °C by making their spore solutions (glycerol 50% - spores suspension (50:50 (v /v)) in cryogenic tubes of 1.8 mL (Nalgene, Fisher Bioblock Scientific, Illkirch, France).

2.2.2 Extraction of ochratoxin A and other fungal secondary metabolites from solid culture medium

The fungus was grown on solid CYA medium for 7 days at 25 °C. Three slices of known weight of the medium colonized by the fungus were taken in an Eppendorf tube and 1 mL of methanol was added into it. The solid medium agarose slices were crushed into small pieces through high speed agitator and incubated at room temperature for 60 min. The mix was centrifuged for 15 min at 13000 rpm and then the supernatant was filtered with the help of filter (Millex® HV 13 mm; Millipore Corporation, Bellerica, MA, USA). The filterate was taken in HPLC vial and was stored at 4° C.

2.2.3 Extraction of ochratoxin A and other fungal secondary metabolites from liquid culture medium

The fungal culture was grown in a liquid synthetic medium (SM) at 25 °C without agitation for 11 days. The liquid culture was filtered through a nitrocellulose membrane of 0.45µm. 15 mL of the filterate was placed in a separating funnel and 100 µL of concentrated HCl was added into it to acidify the culture medium which improves its settling. Mix by inverting the bulb of the funnel time to time, and then 15 mL of chloroform was gently added. Mix again several times by inversion, the gas was removed by opening the cap. The mixture was left to settle down for several minutes (let the cap of the funnel open). The chloroform phase (lower phase) was recovered in a flask and evaporated through a Rotavapeour at 50 ° C with a slight rotation of 150 rpm. The extract was taken with 1 mL methanol and then filtered through a filter (Millex® HV 13 mm; Millipore Corporation, Bellerica, MA, USA) and conserved in a HPLC vial at 4 ° C.

2.2.4 Fluorescence and UV based HPLC Analyse

The HPLC apparatus (High Performance Liquid Chromatography) allows a detection by fluorescence (λex = 332 nm; λem = 466 nm) and by UV (Diode array detector). The system is also fitted with a pump to obtain gradients of solvents. Software (Kroma 3000) can govern the whole system and ensure the acquisition of data. The analytical column used is Uptisphère 5 C18 ODB (150 x 4.6 mm) type with a pre-column of 10 x 4 mm

grade. The mobile phase was a mixture of acetonitrile (B) / 0.2% acetic acid (A). The flow rate was of 1 mL min^{-1} and the column temperature was kept at 30 ° C. The injections were made with an auto-sampler injector (BIO-TEK, Milan, Italy) and the injection volume was 80 µL. For the analysis or the separation of metabolites, a long program of 45 minutes was used (table 3). For quantification of metabolites, standard curves were made from standard metabolites. The minimum detection limit for most metabolites were 20 ng.

Table 3. Long program of 45 min for the separation of mobilities through HPLC.

Solvant / Temps (min)	A	B
0	90	10
30	50	50
35	10	90
43	10	90
45	90	10

2.2.5 Fungal nucleic acid extraction

a. Preparation of fungal material

Spores suspension (final concentration of 10^6 spores / mL) was inoculated in an Erlenmeyer flask (250 mL) containing liquid synthetic medium (100 mL) and incubated at 25 ° C without agitation for 3 days. The liquid culture was then filtered through a nitrocellulose membrane of 0.45µm. The fungal material was recovered, grounded in liquid nitrogen and stored at -80 ° C for subsequent extraction of nucleic acids.

b. Genomic DNA extraction

The DNA molecules were released by lyses of the fungal cell wall and cell membrane. The RNA and proteins are processed by the RNase A and proteinase K (Promega, Charbonnieres, France) respectively. The genomic DNA is precipitated by absolute

ethanol and re-suspended in ultra high quality water. We utilized the following three methods for genomic DNA extraction;

i. High molecular weight DNA extraction

Mycelium was rinsed several times with distilled water, excess water was removed and one gram of mycelium was frozen in liquid nitrogen and then grounded by mortar and pestle. The DNA extraction was performed in a 250 mL Erlenmeyer 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. After incubation for 72 hours at room temperature with shaking (rotary shaker at about 1 cycle per sec) the samples were centrifuged at 2000 x g for 15 minutes and then supernatant was collected. The DNA was then purified by extraction with 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. After phenol chloroform extraction the DNA in the supernatant was precipitated (in the form of a clot) with 0.6 volume of isopropanol. The DNA clot was removed with the help of a sterile pasture pipette, rinsed gently with 70% ethanol, dried in a dessicator and dissolved in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0).

ii. CTAB method

The extraction of DNA involves the preparation of a lyses solution or extraction buffer whose composition is as: CTAB (1%), EDTA (pH 8, 20 mM), NaCl (1.4M), Tris-HCl (pH 8, 100mM) and adjust volume with ultra pure H_2O. The extraction buffer was sterilized through a 0.22 µm filter (Millipore). The mycelium was grounded in liquid nitrogen. To about 300 mg of grounded mycelium, 700 µL of extraction buffer were added and vigorously mixed, then incubated at 50 ° C for 10 minutes and finally transferred to 4 ° C for one hour. Equal volume of phenol-chloroform-isoamyl alcohol (v / v) were added and inverted vigorously 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. To remove residual phenol, equal volume of chloroform (v / v) was then added. A centrifugation was again performed for 5 min at 13,000 rpm. The upper phase

was recovered and transferred to a new Eppendorf tube. 95 % ethanol was added incubated at -20 ° C overnight to precipitate the DNA. A centrifugation for 15 min at 13,000 rpm permitted to have the DNA in a pellet, which was collected and was rinsed with 50 µL of 70 % ethanol to remove salts by centrifugation for 5 min at 13,000 rpm. The pellet in the Eppendorf tubes was air dried and re-suspended in 50 µL of ultra pure sterile water.

The quantity and purity of DNA was checked by measuring optical density (OD) in a spectrophotometer. Finally the DNA was visualized by horizontal agarose gel electrophoresis.

iii. Quick method of DNA extraction

A small quantity of mycelium taken with the help of a sterile pipette tip was grounded in 500 µL of lyses solution (whose composition is; Tris HCl pH 7.4 (400 mM), EDTA (60 mM), NaCl (150 mM), SDS (1 %) and H_2O ultra pure) with the same tip. The mix was incubated for 10 minutes at room temperature. Then 150 µL of potassium acetate buffer (composition for a final volume of 25 mL is; 5M Potassium acetate 15 mL, Glacial acetic acid 2.87 mL and distilled water 7.12 mL) was added into it, vortexed 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. Both were mixed by inversion and centrifuged for 2 minutes 12,000 rpm. The supernatant was removed and the pellet was washed with 300 µL of 70 % ethanol. A centrifugation was performed for 1 minute at 12,000 rpm. The supernatant was removed and the DNA present in the pellet was resuspended in 50 µL of sterile water.

c. Total RNA extraction

The manipulation of RNA is very sensitive due to the presence of RNA destructive enzymes i.e. RNases, in the environment (materials, skin, straw ...). Therefore, precautions must be taken to eliminate RNases (through sterilization of experimental equipment or washing these with 4N NaOH then with water-DEPC (Diethyl

Pyrocarbonate, Sigma) using processed solutions without RNase, pipette tips, filter, gloves). The extraction of RNA is performed using a kit (Tri Reagent, Genomed) and the protocol is as follow:

300 mg of mycelium was grounded with an ultra-Turax in 1 mL of Tri Reagent, incubated for 5 min at room temperature. 200 µL of chloroform were added into it, vortexed for 15 seconds and leave 2 to 3 min at room temperature. A centrifugation at 4 ° C for 15 min at 13,000 rpm was realized and the upper phase was collected. 500 µL of isopropanol was added, mixed and then centrifuged at 4 ° C for 20 min at 13,000 rpm. The supernatant was discarded and 1 mL of 75% ethanol was added to the pellet. A centrifugation at 4 ° C for 5 min at 13,000 rpm permitted to discard the supernatant and to pellet RNA. Re-suspend the air dried RNA in 50 µL of DEPC sterile water.

d. Quantification of DNA and RNA

Optical Density (OD) quantification was performed in a quartz vessel at two different wavelengths at 260 nm, the wavelength of nucleic acids absorption and 280 nm, the wavelength of proteins absorption. The purity of DNA samples can be estimated by the ratio of OD (OD260 nm/DO280 nm). If the ratio is less than 1.6, there could be a contamination of protein and if it exceeds 1.9, there could be a contamination of RNA in the sample.

The amount of DNA is given by the following formula:

[DNA] = OD260 nm × 50* × dilution factor
* 1 unit of OD 260 nm = 50 µg / mL DNA

To quantify RNA the same formula is used, but the factor 50 is replaced by a factor of 40 because 1 unit of OD260 nm = 40 µg /mL RNA.

2.2.6 Horizental agarose gel electrophoresis

Due to the electric field present in the horizontal electrophoresis tank, nucleic acids move through the pores in the agarose gel from the cathode to the anode. 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

The buffer 1X TAE: Tris-Acetate, EDTA (acid Ethylene Diamine Tetra-Acetic) was used both for migration and the preparation of gel. The salt in the buffer allowed conduction of the electric field in the horizontal electrophoresis tank. We used a 0.8 % to 2% agarose gel. The powdered agarose was heated to boil in a microwave in 1X TAE buffer. Then 2 µL of ethidium bromide (BET) were added into it 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. The size marker: 1Kb DNA Ladder (Promega) was used as a reference to estimate sizes of different fragments of nucleic acid.

b. Vizualisation of the gel after electrophoresis

BET is a mutagen which inters to fix itself between the bases of nucleic acids. It allows to visualize the bands in its fluorescence under UV (254 nm). BET should be used with great caution, it is highly carcinogenic. After migration, the gel is analyzed by using an automated trans-illuminator (Quantity one 3-D analyzer, BioRad).

2.2.7 PCR (polymerase chain reaction)

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 (Stratagene 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 suplied by Invitrogen (Cergy Pontoise, France) was utilized to prepare the reaction mix. This kit is composed of an enzyme i.e. Taq DNA polymerase, $MgCl_2$ and 10 X PCR buffer. The reaction was composed of:

Primer 1 (50 µ M)…… 1 µL
Primer 2 (50 µM)……..... 1 µL
dNTP (10 mM chacun)……1 µL
$MgCl_2$ (25mM)……..……1.5 µL
10 X PCR buffer...…….….5 µL
Taq DNA polymerase 5U/µL ……0.5 unit
DNA…… ~ 200 ng
Sterilized H_2O …….qsp 50 µL

The PCR reaction was programmed as follow:

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

Second step: 33-35 cycles

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

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

2.2.8 Reverse transcription –PCR

Reverse transcription (RT) - PCR is a technique which is used for studying gene expression. There are two basic steps to follow the transcriptional signals of a gene i.e. reverse transcription and PCR.

Protocol:

[†] T depends on the primer melting temperature
[‡] t depends on the size of the fragment to amplify

A kit was used for performing RT-PCR. This kit was composed of a reverse transcriptase: MMLV reverse transcriptase, DNase inhabitor (40 U/µL), oligodT (20 µM), dNTP and 5 X buffer (Kit : Advantage, Clontech, BD Biosciences).

A microcentrifuge tube was prepared with RNA (5 µg), OligodT (1 µL) and Sterile 0.01 % DEPC- water (qsp 10 µL). This mix was incubated for 5 min at 70 °C, kept on ice and then 5X buffer (4 µL), dNTP (RT 100mM, 2 µL), DNase nhibitur (1 µL) Sterile 0.01 % DEPC- water (1 µL) were added into it.

The mix was incubated for 5 min at 37 °C. 1 µL of Rtase enzyme was added and incubated for 1 h at 42 °C, followed by 15 min at 70 °C. Finally 60 µL of sterile 0.01 % DEPC- water were added into it.

The complementary DNA (cDNA) thus obtained was then amplified by PCR. During the PCR three control reactions were also performed. Two controls involving the utilization of β-tubuline primers giving a fragment of 450 bp (gene expressed in all conditions) with cDNA and genomic DNA as nuleic acid sours. The third control was the negative control (without cDNA); this permitted to monitor genomic DNA contaminations.

2.2.9 Cloning

a. Principal of cloning

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 a solid LB medium. The bacteria will divide itself and will form a colony population of identical cells containing the DNA fragment inserted in the starting plasmid vector.

b. Preparation of Petri dishes containing LB agar

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

c. Cloning protocol

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

- TOPO-pCR2.1 vector
- Competentes 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

To perform ligation add to a sterile Eppendorf tube (0.2 mL) TOPO-pCR2.1 (1μL), DNA fragment (PCR product or gel purified DNA fragment; 4 μL) and Saline solution (1μL). Mixed gently and incubated for 30 min at room temperature.

e. Transformation

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

f. Analysis and conservation of clones

After incubation, the white clones (take 10 clones) were analyzed by PCR using the primer pair M13 Forward / Reverse M13. Then cultures of the positive clones were made

in liquid LB medium containing ampicillin (50 µg / mL). The recombinant clones were conserved in glycerol at a final concentration of 25 %, in cryogenic tubes of 1.8 mL (Nalgene, Fisher) and stored at -20 ° C.

g. Mini preparation of plasmid DNA

Mini-preparation of plasmid DNA was made by using a kit (RapidPURE ™ Plasmid Mini Kit) according to the protocol described by the supplier.

2.2.10 Transformation vectors formation and transformation of *A. westerdijkiae*

a. Formation of transformation vector by classical method

A 2 kb fragment of the gene of interest was cloned into the plasmid pCR2.1-TOPO (Figure 30). Besides the hygromycin B phosphotransferase (*hph*) cassette of resistance from *Escherichia coli* was generated from plasmid pID2.1 (Tang *et al.*, 1992) and was

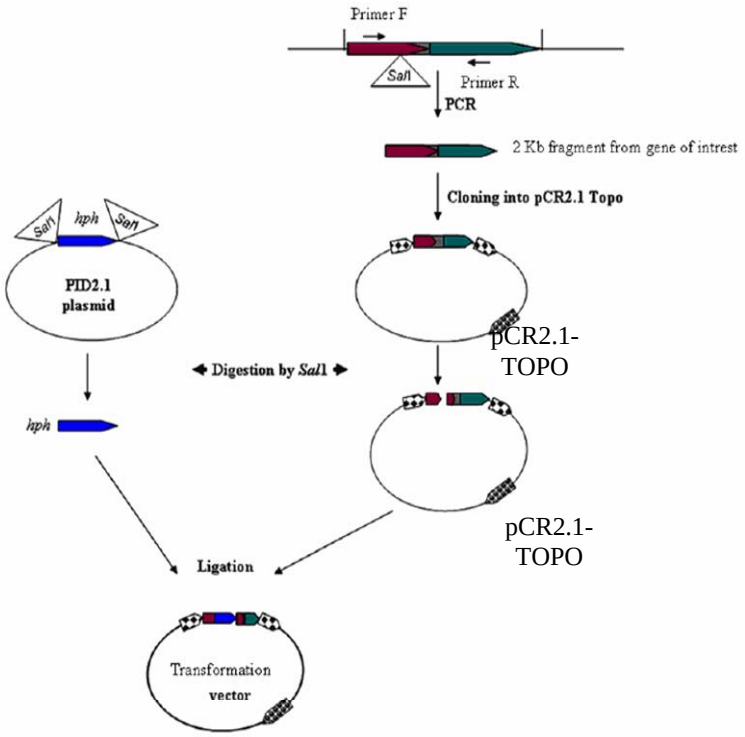


Figure 30. Plasmid based transformation vector formation

inserted into the plasmid pCR2.1-TOPO 2 kb fragment thanks to the *Sal*1 digest to form the transformation vector. The final transformation of *A. westerdijkiae* was performed by fusion of transformation vector with protoplasts of *A. westerdijkiae*.

b. Formation of transformation vector by modified double joint PCR

This new method is based on the approach of double joint PCR (Yu *et al.*, 2004b) and the technique of PCR- mediated generation of gene disruption construct (Kuwayama *et al.*, 2002). This method is composed of three rounds of PCR (Figure 31).

- **First Round PCR: Amplification of 5′ and 3′ regions of the gene of interest (with a portion of *hph*) and cassette of *hph*.**

A fragment of 0.65 Kb 5′-flanking fragment from the gene of interest with a small tail of *hph* was amplified by using primers " 3 " i.e. 25 bases from 5′ -end of the gene of interest, and " 4 " i.e. the reverse complement of 25 bases from 5′-end of *hph* with the reverse complement of 25 bases of 3′ -end of the 5′-flanking gene of interest (Fig. 31, 1st round). Then the 0.65 Kb fragment of 3′ flanking of the gene of interest with *hph* tail was amplified by using primers “5” i.e. 25 bases from 3′ -end of *hph* with 25 bases from 5′-end of the 3′ flanking gene of interest, and "6" i.e. reverse complement of 25 bases from 3′ -end of the 3′ flanking gene of interest (Fig. 31, 1st round). At the end *hph* cassette was amplified by using primers "1" i.e. 25 bases of 5′-end of *hph*, and primer "2" i.e. reverse complement of 25 bases from the 3′ -end of *hph* (Fig. 31, 1st round). The products of the 1st round PCR were analyzed on 0.8 % agarose gel electrophesis.

- **Second round PCR: Fusion of *hph* cassette with 5′ flanking of the gene of interest containing *hph* tail**

The 5′ flanking fragment of gene of interest containing *hph* tail obtained after the first round PCR, was fused with the *hph* cassette (2.414 kb). The principal of this round was that *hph* cassette (the product of the first round) served as template DNA, 5′ flanking fragment of the gene of interest containing *hph* tail (the product of the first round) served as a forward primer while "2" was used as reverse primer (Fig. 31, second round). This reaction was performed using Long Expand Polymerase Roch (specially recommended

for the amplification of fragments larger than 10 Kb). The product of this round PCR was analyzed on 0.8% agarose gel electrophesis.

- **Third round PCR : Fusion of second round PCR product with 3´ flanking of the gene of interest containing *hph* tail**

The 3´ flanking fragment from the gene of interest containing *hph* tail obtained from first round amplification was fused with *hph* cassette flanked by 5´ flanking fragment of the gene of interest (the fragment obtained from the second round) (Fig. 31, 3rd round). In this reaction, the fragment obtained from the second round was used as template DNA,

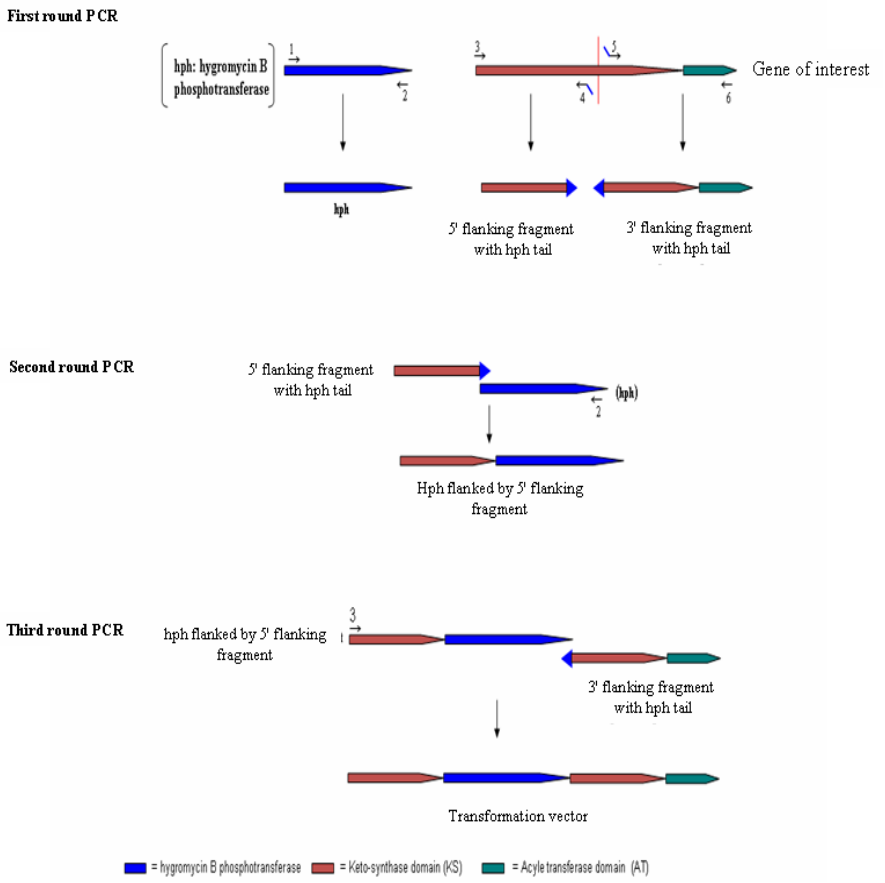


Figure 31. Method of modified double joint PCR. Different colors indicate different functional domains on the gene of interest. Numbers indicate different primers used.

the 3´ flanking fragment from the gene of interest containing *hph* tail served as reverse primer, while "3" served as forward primer. This reaction was performed using Long Expand Polymerase Roch (specially recommended for the amplification of fragments longer then 10 Kb). The product of this round PCR was analyzed on 0.8 % agarose gel electrophesis.

The final product obtained was cloned into plasmid PCR TOPO2.1 to form the transformation vector. The transformation is done by fusion of transformation vector with protoplasts of *A. westerdijkiae*.

c. Complementation vector formation

To construct the complementation vector, the total available fragment of the target gene amplified. The amplified fragment was then either directly used for transformation or cloned into pAN8-1 plasmid containing phleomycine resistance gene cassette and then used for transformation.

d. Formation of protoplasts

For the preparation 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 h until the germination of spores (microscopic verification) at 30 ° C under agitation (125 rpm). The newly germinated mycelium was recovered by filtration on muslin paper (miracloth, Calbiochem) and then washed with 200 mL of solution 1 (0.6M $MgSO_4$). Muslin paper containing mycelium was dried between sterile paper towels and dried mycelium was weighed in a sterile Petri dish. The mycelium was then transferred into a tube (15 mL) 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). Lysing enzyme was added and then incubated on ice for 5 min. The preparation was then placed in shaking (100 rpm) at 28 °C 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 enzyme is 40 mg / mL.

e. Purification of protoplasts

To the protoplasts suspension one volume of separation buffer A (0.6 M sorbitol, 100 mM Tris-Cl, pH 7.0) was added and centrifuged at 3000 rpm for 15 min. Protoplasts were recovered from the interphase in 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). The protoplasts were collected by centrifugation (2600 rpm for 10 min) in the pellet and then a second wash was performed with 10 mL of separation buffer B. The protoplasts were finally suspended in 0.2 mL solution 7 (1.0 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5). The concentration of protoplasts was determined and adjusted to 2 x 10^7 protoplasts / mL with solution 7.

f. Transformation

About 6 µg of the prepared transformation vector (digested by Not1) were added in 150 µL of protoplasts solution (2 x 10^6 protoplasts / mL). Then 50 µL of solution 8 (60% PEG, 10mM $CaCl_2$, 10mM Tris-HCl, pH7.5) were added into it. The mix was gently inverted a few times and then incubated on ice for 20 min. 500 µL of solution 8 were added and then incubated for 20 min at room temperature. 500 µL of solution 7 were added to the mix and was then gently suspended in 30 mL of overlay medium (0.3 % agar YES). The mix was then distributed in 10 Petri dishes containing solid YES medium. All the plates were incubated at room temperature for one day. About 3 mL of 0.3 % agar YES medium containing 150 g / mL of hygromycin B (for transformants) and 120 g / mL of phleomycin (for complemented transformants) were added to each Petri dish. The dishes were then incubated at 30 ° C for 4 days until the appearance of colonies. The colonies were then subcultured several times on fresh solid YES medium supplemented with hygromycin B or phleomycin on Petri dishes.

The genetically complemented mutants without selection cassette were initially screened by growing in Petri dishes containing YES medium and overlayed by about 3ml of YES

0.3 % agar medium (Figure 32). Each of the individual grown after 48 hours of incubation was divided into two halves. One part of the divided colony was transferred to YES without hygromycine and the other part to YES with hygromycine. The successfully complemented mutants lost their effency to grow against hygromycine. These mutants were selected for further screening through PCR and southern hybridization.

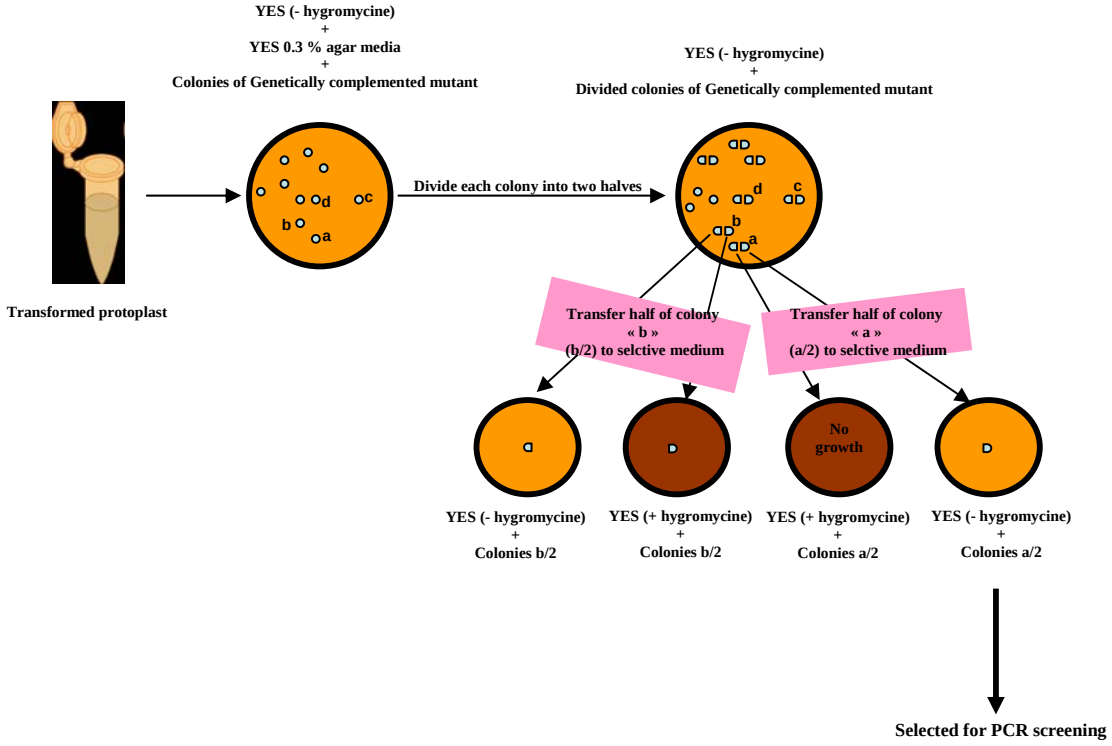


Figure 32. Initial screening of complemented mutants without selection marker.

2.2.11 Confirmation of gene disruption and analyses of the mutants through PCR and Southern hybridization

The transformation efficiency was first 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) and then confirmed through Southern hybridization.

Southern hybridization

Localization of particular sequences within genomic DNA is usually accomplished by the transfer techniques described by Southern (1975). Genomic DNA is digested with one or more restriction enzymes, and the resulting fragments are separated according to size by electrophoresis through an agarose gel. The DNA is then denatured *in situ* and transferred from the gel to a solid support (usually nitrocellulose filter or nylon membrane). The relative positions of the DNA fragments are preserved during their transfer to the filter. The DNA attached to the filter is hybridized to radiolabelled DNA, and autoradiography is used to locate the positions of bands complementary to the probe.

Protocol

a. Restriction digestion of genomic DNA

Steriled pipette tips were cut off for handling high-molecular-weight genomic DNA. 10 µg of genomic DNA was digested in 400 µl total volume with 3 units of restriction enzyme per µg DNA at 37°C for overnight. 0.1 volume (10 µl) of 3 M sodium acetate was added into it and mixed well. Then 2.5 volumes ice-cold 100 % ethanol was added to precipitate DNA at -80 °C for 1 hour. The mix was centrifuged at high speed for 20 minutes. The supernatant was discarded and the pellet was air-dried for 5 minutes and Resuspend in 15 µl 10 mM of Tris pH 7.5. To each sample 3 µl 6 x DNA-loading buffer was added to obtain a final volume of 18 µl. The DNA was migrated and vizualised through electrophesis at 40 Volts, 35 mAmps for 6-16 hours. For partial fragmentation of large DNA fragments (>10 kb) prior to the transfer, leave on UV light box for 10 minutes.

b. Southern transfer to Nylon membrane

Just prior to the transfer, the Nylon membrane (Amarsham, France) was floated on the top of distilled water to wet thoroughly. The lower left-hand corner was cut off for orientation. Denaturation was performed by soaking the gel in at least two gel volumes (500 ml) of 1.5 M NaCl / 0.5N NaOH for 2 x 15 minutes**.** Transfer was set up in a large electrophoresis tray as described in Figure 33. Transfer buffer i.e. 10 x SSC (composition

of 20 x SSC is NaCl (3 M), Sodium citrate (0.3 M) and EDTA (1 mM), was filed into the two tray (transfer tank). The following items (gel-sized, saturated with transfer buffer) were placed on the middle support. All air-bubbles were carefully smoothed-out with a glass pipet or gloved fingertip in each layer seperately.

The transfer system was organized from down to top as followes (Figure 33);

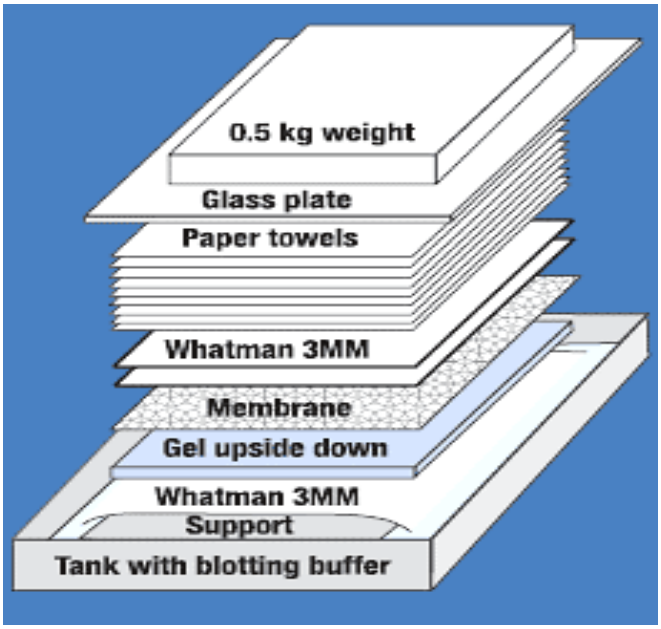


Figure 33. Southern transfer to nylon membrane

- Wick (3mm paper); this should be the same width as your gel and long enough to drape well into the transfer solution.
- 3 pieces of Whatman 3MM paper
- Gel (upside down)
- Nylon membrane (Always handle with clean gloves and blunt-ended forceps. Do not adjust the Nylon membrane once it is placed on the gel)
- 3 pieces of Whatman 3MM paper
- Dry 5 cm stack of paper towels
- Glass plate with weight (ca. 500 g)

The gel was surrounded with plastic wrap or parafilm to prevent the paper towels to come in contact with the wet paper below the gel. The evaporation of the transfer solution was prevented by sealing with plastic wrap or parafilm on either end of the tray. The transfer was performed overnight. Next day, the blotting material was taken off and position of the wells were marked with very-soft-lead pencil. The gel was soaked in 5 x SSC for 5 minutes to remove bits of gel or particles from membrane. The wet membrane was then placed on a paper towel of equal size. The DNA was immobilized by UV-crosslinking (100 mJ / cm^2, push "optimal cross-linking"). The membrane was dried thoroughly at room temperature before placing in prehybridization solution.

c. Prehybridization

Membrane was placed in hybridization bottle with 20 ml (min. 100 µl/cm^2) of prehybridization buffer (Dionized formamide (50 %), 20 x SSC stock solution (6 x), 50 x Denhardt's reagent (Bovine serum albumin (2 %), Ficoll (2 %) and Polyvinylpyrrolidone (2 %)) (5 x), 20 % SDS stock solution (0.5 %), Salmon Testes DNA (Sigma D-9156) (100 µg/ml), dH_2O ad 20 ml) (nylon mesh was used as a spacer before rolling). The setup was incubated in hybridization oven at 42°C for 1-2 hours. Alternatively, without formamide, incubate at 68°C.

d. Radioactive labeling of probe

1-10 µg of target cDNA from vector with restriction digestion was separated through agarose gel electrophoresis. Gel extraction of appropriate band was performed and the fragment was resuspended in 10 mM Tris. 25-50 ng of cDNA-probe was labelled with ^{32}P using the Random Primer Labeling Kit (Amarsham, France). This kit was supplied with;

- Random 9-mer primers
- 5 x *dATP buffer (for use with [*a*-^{32}P]dATP) containing dGTP, dCTP, dTTP, or 5 x *dCTP buffer (for use with [*a*-^{32}P]dCTP) containing dGTP, dATP, dTTP
- [*a*-^{32}P]dATP) or [*a*-^{32}P]dCTP)

- Exo(-) Klenow fragment of DNA polymerase I (5 U/µl)
- Stop mix (0.5M EDTA, pH 8.0)

Procedure

To a microfuge tube the following was added as;

- 25-50 ng (1-23 µl) of template DNA
- 0-23 µl dH_2O
- 10 µl primer (diluted: 10 µM or 10 ng / µl)

The reaction tube was boiled in a water bath for 5 minutes at 100 °C and then centrifuged briefly at room temperature to collect condensed liquid from cap of tube. Now the following reagents were added to the reaction tube;

- 10 µl of 5 x primer buffer (either 5 x *dATP or 5 x *dCTP buffer)
- 5 µl of labelled nucleotide (either [*a*-^{32}P]dATP) or [*a*-^{32}P]dCTP)
- 1 µl Exo(-) Klenow enzyme (5 U/µl)

The reaction components were mixed thoroughly with pipet tip and incubated at 37 – 40 °C for 2 - 10 minutes. The reaction was stopedby addition of 2 µl of stop mix. The primer was purified with Qia-quick PCR Purification Kit (Qiagen, France). Then the radioactivity of the probe was counted. 1 µl of the probe was taken, diluted with appropriate amount of scintillation fluid, use same amount of scintillation fluid as negative control. You should have 1-5 x 10^6 cpm per ml hybridization reaction (20-100 x 10^6 cpm). Finally the probe was denatured by boiling 5 min and chill on ice immediumtely before use.

e. Hybridization

The denatured probe was added to prehybridization solution, incubated overnight (12-24 hours) at 42 °C (or at 68°C without formamide) in hybridization oven.

f. Wash

The membrane was soaked twice for at least 15 minutes each with 100 ml of 7 x SSC / 0.1-0.5% SDS at room temperature. This step was performed in hybridization oven rotating at very low speed. The membrane was then soaked twice for at least 15 minutes each in 100 ml of 1 x SSC / 0.5-1% SDS at 37°C. This step was also performed in hybridization oven rotating at very low speed. Finally soaked for 1 hour in 100 ml of 0.1 x SSC / 1 % SDS at 68°C. Radioactivity was checked with Geiger-counter.

g. Detection

After washing, the membrane was bloted with filter paper (Whatman 3MM) to remove most of the excess moisture. Moist blots were wraped in a plastic wrap prior to autoradiography. Finally it was exposed to X-ray film with an intensifying screen at -80°C for 1 to 6 days.

Results and Discussion

3- Results and Discussion

Previously in our laboratory Atoui *et al*; (2006) identified several KS domains from different PKS genes in *Aspergillus westerdijkiae* (*A. ochraceus*) and *A. carbonarius*. They investigated the diversity of these domains and found that these domains were distributed in 5 different clusters on the phylogenetic tree, indicating that they most probably represent polyketide synthases responsible for different functions (Figure 34). Among these domains we have initially selected 4 KS domains including *aoks1, aolc35-12, aomsas* or *aolc35-2* and *aolc35-6* for their genetic and functional characterization. According to Atoui et al, (2006) *aoks1* and *aolc35-6* could belong to the nonaketide group of PKS genes, *aolc35-12* to diketide and *aomsas* to MSA group of PKS genes.

In the first article included in this section we described the characterization of a 2 kb partially sequenced *aoks1* gene. This gene was found to have a high level similarity with other PKS gene including lovastatine biosynthesis gene "*lovb*" in *A. terreus*, compactin biosynthesis gene "*mlcA*" in *Penicillium citrinum* and ochratoxin A biosynthesis gene "*otapksPN*" in *P. nordicum*. *aoks1* gene knockout mutants "*ao*Δ*ks1*" were produced by the insertion of *Escherichia coli* hygromycin B phosphotransferase gene cassette in the genome of *A. westerdijkiae. ao*Δks1 mutants were found deficient in the biosynthesis of ochratoxin A (OTA) but still producing mellein and hydroxymellein which was previously proposed as intermediate of OTA.

In the second article characterization of a 3.22 kb partially sequenced *aolc35-12* gene has been described. The acyl-transferase domain of this gene was found to be an analogue of a previously characterized OTA-PKS gene in *A. ochraceus* (O'callaghan *et al*., 2003). Further *aolc35-12* has shown similarities of 60% to an OTA-PKS gene in *A. niger* while 34-40 % similarities with other pks gene in *Penicillium marneffei, A. clavatus, A. terreus,* and *A. fumigatus*. Disruption of *aolc35-12* gene in the genome of *A. westerdijkiae* revealed that this gene is also important for OTA biosynthesis but not for mellein. These results once again confirm that mellein has no role in OTA biosynthesis. Revers transcription PCR studies of the two OTA-PKS genes of *A. westerdijkiae* in the two OTA

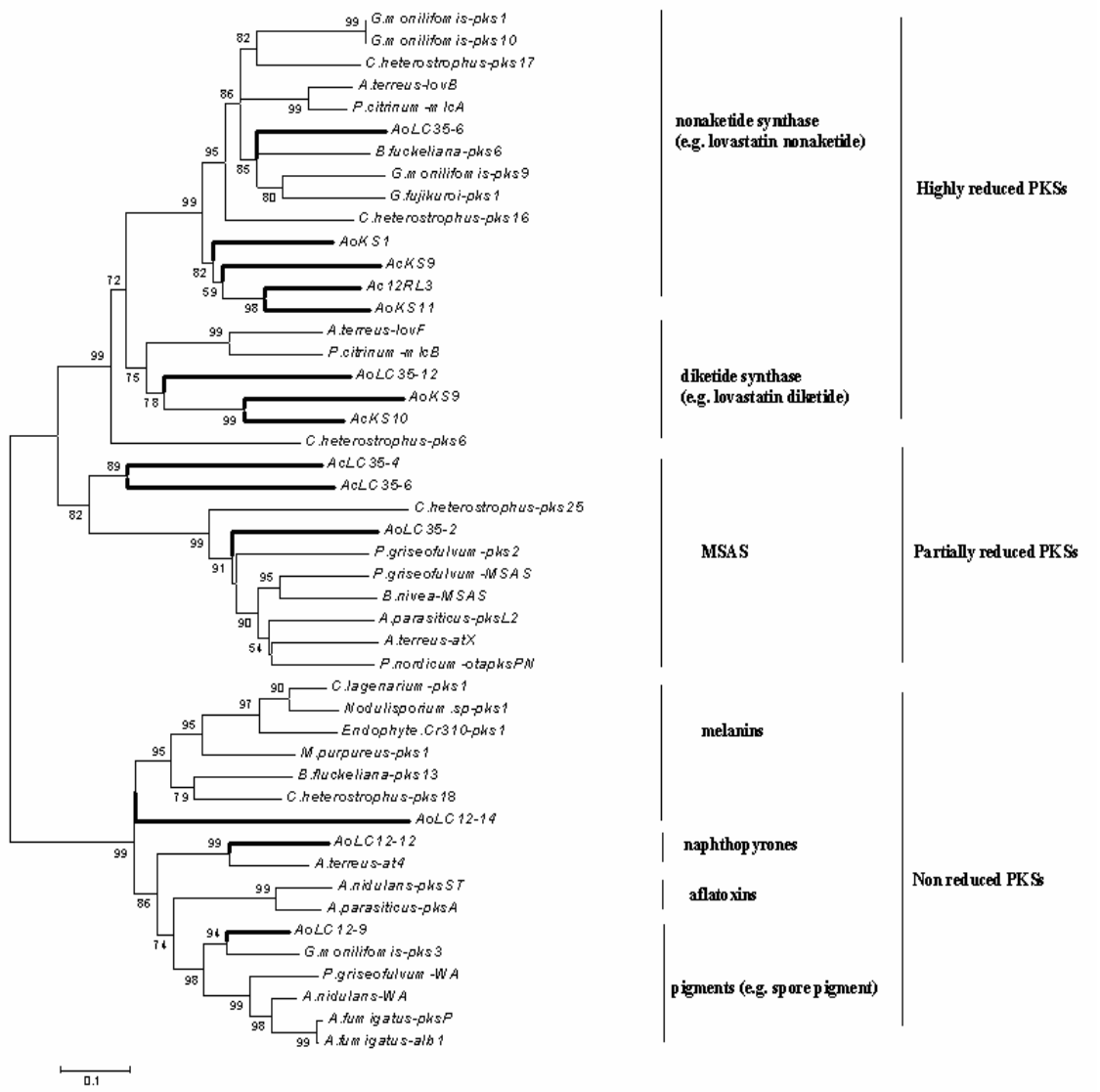


Figure 32. Neighbor-joining phylogenetic tree of KS sequences of fungal PKSs

negative mutants i.e. *ao*Δks1 and *ao*Δ*lc35-12*, shown that *aolc35-12* differentially controled the expression *aoks1* gene.

The third article explains the characterization of a complete 5.298 kb *aomsas* gene. This gene displayed high degree similarity with different methylsalicylic acid synthase genes found in *Byssochlamys nivea*, *P. patulum*, *A. terreus* and *Streptomyces viridochromogenes*. Based on reverse transcription PCR and kinetic secondary metabolites production studies, *aomsas* was found to be associated with the biosynthesis of two lactonic metabolites of *A. westerdijkiae* i.e. isoasperlactone and asperlactone. This association was confirmed by disruption of *aomsas* gene through insertional inactivation. The obtained mutants were deficient in the biosynthesis of isoaspelactone, asperlactone and 6- methylsalicylic acid. Through chemical complementation experiments 6-methylsalicylic acid, diepoxide and aspyron were identified as important intermediates in the biosynthetic pathway of isoasperlactone and asperlactone.

The fourth article deals with the characterization of a partially sequenced 1.999 kb *aolc35-6* gene. This gene displayed a high degree identity to different hybrid *PKS-NRPS* genes of *A. clavatus*, *A. niger* and *Magnaporthe grisea* and a slight similarity to *msas* type *PKS* gene of *A. westerdijkiae*. A mutant of *A. westerdijkiae* "*aoΔlc35-6*", in which *aolc35-6* gene was disrupted, lost their capacity to produce three important polyketide metabolites penicillic acid, isoasperlactone and asperlactone and two intermediates orsellinic acid and 6-methylsalicylic acid. Chemical complementation study confirmed the importance of orsellinic acid and 6-methylsalicylic acid in the biosynthetic pathway of penicillic acid and isoasperlactone / asperlactone respectively. We further demonstrated that *aolc35-6* differentially controls the expression of the first isoasperlactone / asperlactone gene "*aomsas*" in *A. westerdijkiae.*

Chapter I

Article 1: Published in Fungal Genetics and Biology (2009), Volume 46, 77-84.

3.1 *Aspergillus westerdijkiae* polyketide synthase gene *"aoks1"* is involved in the biosynthesis of ochratoxin A.

Nafees Bacha[(1)], Ali Atoui[(1,2)], Florence Mathieu[(1)], Thierry Liboz[(1)] and Ahmed Lebrihi[(1)§]

[1] Département Bioprocédés et Systèmes Microbiens, Laboratoire de Génie Chimique UMR5503 (CNRS/INPT/UPS), Ecole Nationale Supérieure Agronomique de Toulouse, Institut National Polytechnique de Toulouse, 1, avenue de l'Agrobiopôle, BP32607, 31326 Castanet Tolosan, France.

[2] Lebanese Atomic Energy Commission-CNRS, P.O. Box 11-8281, Riad El Solh, 1107 2260 Beirut, Lebanon.

[§] Corresponding author

Tel: 00 33 (0)5 62 19 39 44

Fax: 00 33 (0)5 62 19 39 01

E-mail: lebrihi@ensat.fr

Abstract

Ochratoxin A (OTA) is a potential nephrotoxic, teratogenic, immunogenic, hepatotoxic and carcinogenic mycotoxin, produced by *Aspergillus westerdijkiae* NRRL 3174. Herein we describe the characterization of a putative OTA-polyketide synthase gene "*aoks1*", cloned by using gene walking approach. The predicted amino acid sequence of the 2 kb clone display 34-60 % similarities to different polyketide synthase genes including lovastatine biosynthesis gene "*lovb*" in *A. terreus*, compactin biosynthesis gene "*mlcA*" in *Penicillium citrinum* and OTA biosynthesis gene "*otapksPN*" in *P. nordicum.* Based on the reverse transcription PCR and kinetic secondary metabolites production studies, *aoks1* expression was found to be associated with OTA biosynthesis. Further a mutant, in which the *aoks1* gene was inactivated by *E. coli* hygromycin B phosphotransferase gene, lost the capacity to produce OTA, but still produced mellein. To our knowledge this report describes for the first time characterization of a gene involved in OTA biosynthesis, with the information about mellein which was proposed in the literature to be an intermediate OTA. This study also suggests that *aoks1* may be the second polyketide synthase gene required for OTA biosynthesis in *A. westerdijkiae* NRRL 3174.

Key Words: *Aspergillus westerdijkiae*, *aoks1*, Ochratoxin A biosynthesis, Mellein, Polyketide synthase.

1. Introduction

Ochratoxin A (OTA) is a polyketide secondary metabolite produced by many *Penicillium* and *Aspergillus* species (Abarca et al., 1994; Dalcero et al., 2002; Teren et al., 1996; Varga et al., 2003). This mycotoxin consisting of a polyketide derived from a dihydroiso-coumarin moiety linked through the 12-carboxyl group to phenylalanine, via an amide linkage (Fig. 1a). It is a nephrotoxin which also displays hepatotoxic, teratogenic, and immunosuppressive properties; and has been classified by The International Agency for Research on Cancer as a possible human carcinogen (category 2B) (Kuiper-Goodman & Scott, 1989; Petzinger & Ziegler, 2000).

Ochratoxin A is a common contaminant of grains such as barley, corn, rye, wheat, and oats, with cereal-based products typically accounting for 50–80% of the average consumer intake of the mycotoxin (Jorgensen & Jacobsen, 2002). OTA has also been reported in other plant products including coffee beans, spices, nuts, olives, grapes, beans, and figs (Batista et al., 2003; Battilani et al., 2003; Bayman et al., 2002; Jorgensen, 1998). The presence of OTA has been detected in a range of beverages (Zimmerli & Dick, 1996) and has also been reported in body fluids and kidneys of animals and humans (Magan & Olsen, 2004).

Aspergillus westerdijkiae NRRL 3174 which is recently dismembered from *A. ochraceus* (Frisvad *et al.*, 2004a), is considered to be the main OTA producer in tropical region. It can produce other important polyketide metabolites including; penicillic acid, asperlactone, isoasperlactone, mellein and hydroxymellein (Atoui *et al.*, 2006; Gaucher & Shepherd, 1968). To the present day not much information are available about the biosynthetic pathway of OTA in any fungal species. Based on a mechanistical model according to the structure of OTA a biosynthetic pathway has been previously proposed, according to which the heterocyclic portion of OTA is structurally similar to mellein (Fig. 1b) (Huff & Hamilton, 1979). Thus mellein has been proposed as a precursor of OTA. In contrary, Harris and Mantle, (2001) described in experiments with labelled precursors of OTA that mellein does not seem to play a role in OTA biosynthetic pathway.

In spite of a remarkable variety of end products, the individual polyketide biosynthetic pathways apparently follow a common basic reaction scheme. The key chain-building

step of this reaction scheme is a decarboxylative condensation analogous to the chain elongation step of classical fatty acid biosynthesis (Birch & Donovan, 1953; Kao *et al.*, 1994). In the biosynthesis of most polyketide metabolites, the successive condensation step of small carbon precursor acid is catalyzed by a group of multifunction enzyme system called polyketide synthases (PKSs), (Metz et al., 2001). A typical fungal PKS is composed of principal domains including ketosynthetase (KS), acyltransferase (AT) and acyl carrier protein (ACP) and optional domains including dehydratase (DH), enoyl reductase (ER), ketoreductase (KR) and thioesterase (TE) (Graziani et al., 2004). Presence or absence of optional domains in a PKS decides about the type of polyketide formed. PKSs producing highly reduced polyketides contain KR, DH and ER optional domains; PKSs producing partially reduced polyketides contain KR and DH optional domains, while PKSs producing non-reduced polyketides contain none of these domains (Fujii *et al.*, 2001; Yu & Leonard, 1995).

Untill now different techniques like genomic DNA bank, cDNA bank and subtractive PCR has been utilized to identify various PKS genes responsible for the biosynthesis of various polyketides (Bridge et al., 1998). Pairs of degenerated primers targeting KS domain; which is the most conserved domain among different PKSs, have been previously designed. These primers were found to have the capability to amplify KS domain fragment from different types of PKS genes (Bingle *et al.*, 1999; Lee *et al.*, 2001a; Liou & Khosla, 2003; Nicholson *et al.*, 2001). In our laboratory these degenerated primers were used to identify 9 different KS domains in *A. westerdijkiae* NRRL 3174 (= *A. ochraceus*) (Atoui et al., 2006), including the KS domain sequence of *aolc35-12* gene (Genbank accession number AY583208). *aolc35-12* gene overlaps the AT domain of the *pks* gene characterized by O'Callaghan et al. (O'Callaghan *et al.*, 2003). The authors demonstrated that disruption of *pks* gene stop the biosynthesis of OTA, without any information about mellein.

In this study, we report the characterisation of a PKS gene (*aoks1*) required for the biosynthesis OTA in *A. westerdijkiae* NRRL 3174. Disruption of this gene stop the biosynthesis of OTA but did not affect the biosynthesis of mellein. This gene is different from the *pks* gene reported by O'Callaghan et al. (O'Callaghan *et al.*, 2003).

2. Materials and methods

2.1. Fungal strain and culture conditions

A. westerdijkiae NRRL 3174 strain provided by Dr. Olivier Puel (Laboratoire de Pharmacologie-Toxicologie, Toulouse France) was grown for sporulation at 25°C on potato dextrose agar for 7 days. Spores were collected using a solution of 0.01% (v/v) Tween 80, counted by using Thoma Bright line counting chamber (Optick labor) , 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 synthetic medium (SAM) at 25 °C for 2 to 18 days, without shaking. The composition of SAM (per liter of distilled water) was: 3 g NH_4NO_3, 26 g K_2HPO_4, 1 g KCl, 1 g $MgSO_4.7H_2O$, 10 mL mineral solution (composition per liter of distilled water: 70 mg $Na_2B_4O_7.10H_2O$, 50 mg $(NH_4)_6Mo_7O_{24}.4H_2O$, 1000 mg $FeSO_4.7H_2O$, 30 mg $CuSO_4.5H_2O$, 11 mg $MnSO_4.H_2O$, 1760 mg $ZnSO_4.7H_2O$), and 50 g glucose. The pH of the medium was adjusted to 6.5 by the addition of 2 N HCl. Mycelium was harvested by filtration through a 0.45µM filter, grounded in liquid nitrogen and then stored at -80 °C before nucleic acid extraction. Secondary metabolites were extracted from filtrates of 2 to 18 days old cultures medium. Three replications of each sample were analyzed.

2.2. Nucleic acid extraction

Rapid method of genomic DNA extraction (Lui et al., 2000) was used for transformants screening by PCR. Large quantity genomic DNA was extracted by CTAB extraction method (Gardes & Bruns, 1993). The quality and quantity of DNA were estimated by measuring OD 260 nm / OD 280 nm and OD 260 nm respectively.

Total RNA was extracted from *A. westerdijkiae* NRRL 3174 using the Tri-reagent (Euromedex France) DNA / RNA/ Protein extraction kit. The quality and quantity of RNA was checked by the OD 260 nm / OD 280 nm ratio and agarose gel electrophoresis according to standard protocols (Sambrook et al., 1989).

2.3. Cloning of aoks1 gene in A. westerdijkiae *NRRL 3174*

Degenarated primers were used to extend *aoks1* gene by gene walking approach. Table 1 list all the primers used in this study. Figure 2 marks position of specific primer AoKS1

(designed from the KS domain fragment of *aoks1* gene) and the two degenerated primers ATR1, ATR2 (designed from the most conserved regions of AT domains of different fungal PKSs). In two consective PCRs; first a 700 bp fragment was amplified using primers AoKS1 and ATR1 and then a 1400 bp fragment was amplified using primers AoKS1 and ATR2. The PCR products were cloned in pCR2.1 plasmid and sequenced. Alignment of the sequenced fragments was performed to search for consensus.

2.4. PCR reaction and sequencing

PCR was performed with the *Taq* recombinant polymerase (Invitrogen, USA). Amplification was carried out in a 50 µl reaction mixture containing: 5 µl of *Taq* polymerase 10× buffer, 1·5 µl of 50 mM $MgCl_2$, 1 µl of dNTP 10 mM of each (Promega), 1 µM of each primer, 1·5 U of *Taq*, about 200 ng of DNA genomic, H_2O up to 50 µl. Reaction conditions were: 94 °C for 4 min, (94 °C for 45 s, 53 °C for 45 s and 72 °C for 1 min) × 30 cycles followed by an incubation at 72 °C for 10 min. The amplified products were examined by 1 % (w/v) agarose (Promega) gel. The PCR products were cloned into pCR2.1-Topo vector (Invitrogen) according to the supplier's instructions. Sequencing of the fragments was performed by Genomexpress (Grenoble, France).

2.5. 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 CLC Main WorkBench (CLC Bio, Denmark)

2.6. RT-PCR reaction

The expression of aoks1 and aolc35-12 genes in A. westerdijkiae NRRL 3174 were examined by using RT-PCR with specific pairs of primers AoKSF / AoKS1R designed from the AT domain of aoks1 gene and LC12F / LC12R designed from the KS domain of aolc35-12 gene (Table 1). For RT-PCR, total RNA was treated with DNase I (Promega) to remove DNA contamination. cDNA was synthesized from each sample with Advantage RT- for-PCR Kit (BD Biosciences) according to the supplier's manual. cDNA

amplification were performed using Taq recombinant polymerase (Invitrogen,USA). Beta tubulin was used as positive control using primers TubF and TubR (Table 1).

2.7. Disruption of aoks1 gene in A. westerdijkiae NRRL 3174

The *aoks1* gene was disrupted by inserting the *E. coli* hygromycin B phosphotransferase gene (*hph*) flanked by *A. nidulans trpC* promoter and terminator sequences (Cullen et al., 1987) from plasmid pID2.1 (Tang et al., 1992). The transformation vector construction is schematically represented in Figure 3a. A 1690 bp fragment of the *aoks1* gene containing the *Sal*I restriction site was amplified by PCR, using the primer pair AoKS1F / AoKS1R (Table 1). This fragment was ligated into pCR2.1-Topo plasmid (Invitrogen) generating the TopoKS plasmid. TopoKS and pID2.1 plasmids were restricted using *Sal*I enzyme. The *hph* cassette (2.4 kb) obtained was ligated with restricted TopoKS plasmid to produce TopoKS*hph* vector, in which the 5` and 3`-ends of *hph* cassette are flancked by *aoks1* gene fragments. *A. westerdijkiae* protoplasts were prepared and transformed with TopoKS*hph* vector as previously described (O'Callaghan et al., 2003) (Fig. 3b). 40 mg/mL lysing enzyme (Sigma) was used for the preparation of protoplasts.

2.8. Screening of the transformants

Hygromycin-resistant transformants were selected on YES medium (20 g/l of yeast extract, 1 M sucrose) supplemented with 150 µg/mL of hygromycin B. Transformant plates were incubated at room temperature for 24 h and then transferred to 30°C for 4 days. Hygromycin resistant transformants were further screened through a PCR, using *hph* gene specific primers *hph*2F and *hph*2R (Table 1). Positive transformants were then subjected to a second PCR using *aoks1* gene specific primer AoKS1F and *hph*2R. Finally the disruption was confirmed through southern hybridization (Southern, 1975), in which total genomic DNA from both wild type and *ao*Δ*ks1* mutant strains were digested with restriction enzyme *Bts*1. This restriction enzyme was choosen because it cut only once in the 5´- flanking region of the 1690 bp *aoks1* fragment (position 599 bp) but does not cut the *hph* cassette or 3´- flanking end of the *aoks1* fragment (Fig. 3b). The digested DNA was then transferred to nylon membrane (Amersham, France) and probed with radioactively labelled DNA fragments of *aoks1* gene "KP" and *hph* gene "HP" amplified

through primer pair AoKSF / AoKS1R and *hph*2F/*hph*2R respectively (probes positions are shown in Fig. 3b).

2.9. Fluoresence study

In order to check the loss of OTA production by the *ao*Δ*ks1* mutant, Czapek Yeast extract Agar (CYA) plates of both wild type *A. westerdijkiae* NRRL 3174 and *ao*Δ*ks1* mutant were incubated for 10 days at 25°C and then illuminated with UV light (265 nm). Production of OTA is characterized by a blue fluorescent halo under UV.

2.10. Extraction of secondary metabolites

For secondary metabolites extraction, 30 mL filtrate sample of the culture medium was acidified with 200 µL of 12 N HCl, mixed with 30 mL chloroform and vigorously shaken for 10 min. The solvent phase was then decanted, dried under vacuum and re-dissolved in 0.5 mL Methanol. 20 µL of the sample was then further analysed by HPLC.

2.11. High-performance liquid-chromatography (HPLC) analysis

The HPLC apparatus consisted of a solvent delivery system, with both fluorescence (λ_{ex} = 332 nm; λ_{em} = 466 nm) and UV detectors (BIO-TEK, Milan, Italy). The analytical column used was a 150 x 4.6 mm Uptisphere 5 µm C18 ODB fitted with a guard column of 10 x 4 mm. The column temperature was 30 °C. Kroma 3000 (BIO-TEK) was the data acquisition system. Injections were done with an auto-injector (BIO-TEK, Milan, Italy) and the injection volume was 20 µL. The samples were analyzed by linear gradient elution using 0.2 % glacial acetic acid in 99.8 % water (v/v) (A) and 100 % acetonitrile (HPLC grade) (B). The crude extract was analyzed using a linear elution gradient over 45 min at a flow rate of 1 ml/min, starting from 10 to 50% solvent B over the first 30 min, continued by a linear gradient to 90 % of B in 5 min, followed by an isocratic flow of 90% solvent B for 8 min, and a return to initial conditions over the last 2 min of the run.

Secondary metabolites of *A. westerdijkiae* NRRL 3174 were detected by comparing the elution time and maximum absorption of UV with the standards (Sigma Aldrich France). All the standards were used at a concentration of 10µg/mL. Mellein (λ_{max} = 214; 245 and 314 nm) and ochratoxin A (λ_{max} = 216; 250 and 332 nm) were released at 27 min and 34 min respectively.

2.12. Sequence accession number

The sequence obtained was deposited in Genbank under the accession number AY583209.

3. Results and Discussion

3.1. Cloning part of aoks1 gene in A. westerdijkiae NRRL 3174

A 700 bp KS domain fragment of *aoks1* gene was previously identified in *A. westerdijkiae* NRRL 3174 (Atoui et al., 2006). With the aim of extending this fragment of *aoks1* gene, two conserved regions **FTGQGAQ** and **AYHSSHM** in the AT domains of different fungal PKSs were used to design two degenerated primers i.e. ATR1 and ATR2 (Fig. 2). These degenerated primers with a specific primer AoKS1 i.e. designed from the KS domain of *aoks1* (Fig. 2), allowed to clone an additional 1300 bp fragment of *aoks1* gene. Atoui et al, (2006) further used the degenerated primers of Bengle et al, (1999) and Nicholson et al, (2001) and identified eight other KS domains of different PKSs in same fungi. The phylogenetic analysis of all the nine KS domain fragments identified in *A. westerdijkiae*, clustured *aoks1* gene with four other PKSs producing reduced polyketides (Atoui et al., 2006). Several mycotoxins such as OTA, viomellein, and xanthomegnin and others polyketides derived secondary metabolites such as mellein produced by *A. westerdijkiae* NRRL 3174 belong to the reduced polyketide groups. Therefore, we focused on the 5 reducing PKS genes including *aoks1* and assumed that these genes could be potential candidates for the biosynthesis of one or more of the reduced polyketides produced by *A.westerdijkiae*.

The predicted amino acid sequence of the 2 kb *aoks1* gene displayed about 60 % identity to nonaketide synthases gene "*lovB*" involved in lovastatin biosynthesis in *A. terreus* (Hendrickson et al., 1999), and compactin biosynthesis gene *mlcA* in *Penicillium citrinum* (Abe et al., 2002) and 34 % identity to OTA biosynthesis gene *otapksPN* in *P. nordicum* (Färber and Geisen, 2004; Karolewiez and Geisen, 2005) (Fig.2). The AT domain of *aoks1* displayed about 39 % identity to the AT domain of *pks* gene involved in OTA biosynthesis in *A. ochraceus* (O'Callaghan et al., 2003). Alignment study also revealed that the *pks* gene characterized by O'Callaghan et al. (2003) overlaps (share more than 98 % identity) a PKS gene *aolc35-12*, previously identified in *A. westerdijkiae* (Fig.2) (Atoui et al., 2006; Dao et al., 2005). This finding confirms that *A. ochraceus* strain used by O'Callaghan et al. (2003) and *A. westerdijkiae* strain used in our study contain similar gene.

3.2. Kinetic production of secondary metabolites in A. westerdijkiae *NRRL 3174 and expression of aoks1 and aolc35-12 genes*

Production of secondary metabolites was followed during the growth of *A. westerdijkiae* NRRL 3174 in synthetic medium (SAM). We observed that biosynthesis of secondary metabolites seemed to be associated with the delayed log phase of fungal mycelial growth (Fig. 4a). This fact has also been realized previously, where biosynthesis of various secondary metabolites were found to be associated with fungal growth and development (Bu'Lock et al., 1968; Calvo et al., 2001; Calvo et al., 2002; Hopwood, 1988). HPLC traces revealed that the two structurally related metabolites mellein (λ_{max} = 214; 245 and 314 nm) and ochratoxin A (λ_{max} = 216; 250 and 332 nm) are released at 27 min and 34 min respectively (Fig. 6b). Both these metabolites were detected from day 4 of fungal growth. Thereafter these metabolites were simultaneously produced to reach a maximum level at day 7 for mellein, and day 12 for OTA (Fig 4a). Later, mellein constantly decreased with time and reached a minimum level at day 16. Small decrease in the production OTA was observed up to day 17 (Fig. 4a).

Expression study of *aoks1* gene showed that the transcriptional signal started from day 3 of *A. westerdijkiae* growth in synthetic (SAM) medium (Fig. 4b). The signal reached its maximum level at day 5 and remained high up to day 10. From expression of aoks1 gene and kinetic production of secondary metabolites we observed that, the low level expression of *aoks1* gene at day 3 and 4 could correspond to the limited production of OTA in the initial few days of fungal growth. During the high transcriptional signal period of *aoks1* gene i.e. day 5 to day 10, production of OTA continuously increased and reached its maximum limit just after the end of this period (Fig. 4a and 4b). These resuts suggest that production pattern of OTA seems to be associated with expression pattern of *aoks1* gene. On the basis of expression studies several other PKS genes producing reduced type polyketides were found to be important for OTA biosynthesis in other organisms like *P. nordicum* (Färber & Geisen, 2004; Karolewiez & Geisen, 2005) and *Penicillium verrucosum* (Schmidt-Heydt et al., 2008).

We also studied the expression pattern of *aolc35-12* gene (similar to *pks* gene involved in OTA biosynthesis, characterized by O'Callaghan et al, (2003)) in *A. westerdijkiae*. Transcriptional signal of *aolc35-12* gene started on day 3 and reached a maximum at day

4 of fungal growth (Fig. 4b). The signals then gradually decreased and disappeared at day 10. O'Callaghan et al. (2003) also stated that expression of *pks* gene appeared strongly at the early fungal growth stage and occurred to a lesser extent at later time points. This suggests that the *pks* gene characterized by O'Callaghan et al. (2003) and *aolc35-12* not only overlap at amino acid level (Fig. 2) but also produce similar expression patterns. We further observed that, although both *aoks1* and *aolc35-12* genes are expressed within the production period of OTA but both produced different expression patterns (Fig. 4b).

3.3. Disruption of aoks1 gene in A. westerdijkiae *NRRL 3174 and secondary metabolites production in ao*Δ*ks1 mutant*

After transformation of *A. westerdijkiae* with TopoKS*hph* vector (Fig. 3b), twenty seven transformants were obtained. These transformants were subsequently screened by two consective PCRs to monitor integration of *hph* cassette in the genome of *A. westerdijkiae*. A fragment of 0.6 kb corresponding to *hph* cassette is amplified using primer pair *hph*2F / *hph*2R in only two out of the twenty seven transformants (Fig. 5a, lane 1). Similarly a 1.2 kb *aoks1* / *hph* shared fragment was amplified when the two positive transformants were subjected to a second PCR using primers AoKS1F and *hph*2R (Fig. 5a, lane 4). No amplification was observed in the wild type *A. westerdijkiae* with any of the primers combination (Fig. 5a, lane 2 and 3). Genomic DNA from wild type *A. westerdijkiae* and the two positive *ao*Δ*ks1* transformants were analyzed by southern blotting. No signal was detected when digested DNA of wild type *A. westerdijkiae* was probed HP (Fig. 5b, lane 1), while a signal corresponding to 2.2 kb was observed when the wild type DNA was probed KP (Fig. 5b, lane 3). On the other hand probing the digested DNA of *ao*Δ*ks1* transformants with KP and HP probes resulted into an expected signal of 4.6 kb i.e 2.2 kb aoks1 fragment + 2.4 kb *hph* fragment (Fig. 5b, lane 2 and 4 respectively).

Both *ao*Δ*ks1* mutant and wild type *A. westerdijkiae* NRRL 3174 were inoculated in solid CYA medium and incubated for 10 days at 25°C. After the incubation period we observed no difference in fungal growth, sporulation, or pigment production. In contrary, *ao*Δ*ks1* mutant lost the characheristic fluorescence of OTA, when observed under UV light (Fig. 6a). This method has been usefully used by O'Callaghan et al. (2003) in order to identify OTA negative mutants.

Further, the two positive *ao*Δ*ks1* mutants were inoculated into liquid SAM medium to check kinetic production of secondary metabolites by using HPLC traces and UV spectra. Disruption of *aoks1* abolished OTA production in the *ao*Δ*ks1* mutant but did not affect the production of an important metabolite mellein (Fig. 6b). Mellein was previously thought to be an intermediate in the biosynthesis of OTA (Huff & Hamilton, 1979). Recently this hypothesis has been dissented by Harris and Mantle (2001) using labbeled precursors of OTA. They found no evidence for the intermediate role of mellein in OTA biosynthetic pathway. This is in agreement with our results, where the disruption of *aoks1* gene has been shown to interrupt the biosynthesis of OTA without affecting the mellein production. Indeed, during the kinetic production study of secondary metabolites by *ao*Δ*ks1* mutant, we observed that mellein accumulation increased after disruption of *aoks1* gene and when OTA production was halted (Fig. 4a and 6c). This could be explained by the fact that mellein and OTA use the same precursor (such as acetyl-CoA), so possibly deletion of one pathway resulted in an increase in the flow of second pathway. In contrary, the study reported by O'Callaghan et al. (2003) about OTA biosynthesis and *pks* gene provides no information concerning the presence or absence of some metabolites like mellein.

We concluded that two different PKS may be involved in the biosynthesis of OTA and that mellein has no role in OTA biosynthesis in A. westerdijkiae NRRL 3174. To date, only three cases have been reported that involve two different fungal PKSs essential for a single polyketide: a set of two unusual type I multifunctional PKSs for the biosynthesis of lovastatin and compactin in *Aspergillus terreus* and *Penicllium citrinum* respectively (Abe et al., 2002; Hendrickson et al., 1999; Kennedy et al., 1999) and two PKS have been reported to be involved in the biosysnthesis of the mycotoxins zearaleone in *G. zea* (Kim et al., 2005) and T toxin in *C. heterosphorus* (Baker et al., 2006).

Acknowledgements

The authors are grateful to the Higher Education Commission of Pakistan for according PhD scholarships to Mr. Nafees Bacha.

References

Abarca, M.L., Bragulat, M.R., Castella, G., Cabanes, F.J., 1994. Ochratoxin A production by strains of *Aspergillus niger* var. niger. Appl. Environ. Microbiol. 60**,** 2650-2652.

Abe, Y., Suzuki, T., Ono, C., Iwamoto, K., Hosobuchi, M., Yoshikawa, H., 2002. Molecular cloning and characterization of an ML-236B (compactin) biosynthetic gene cluster in *Penicillium citrinum*. Mol. Genet. Genomics 267**,** 636-646.

Atoui, A., Dao., P., Mathieu, F., Lebrihi, A., 2006. Amplification and diversity analysis of Ketosynthase domains of putative polyketide synthase gene in *Aspergillus ochraceus* and *Aspergillus carbonarius* producers of ochratoxin A. Mol. Nutr. Food. Res. 50**,** 448-493.

Baker, S.E., Kroken, S., Asvarak, T., Inderbitzin, P., Li, B.Y., Shi, L., Yoder, O.C., Turgeon, B.G., 2006. Two polyketide synthase-encoding genes are required for biosynthesis of the polyketide virulence factor, T-toxin, by *Cochliobolus heterostrophus*. Mol. Plant. Microbe. Interact. 19**,** 139-149.

Batista, L. R., Chalfoun, S.M., Prado, G., Schwan, R.F., Wheals, A.E., 2003. Toxigenic fungi associated with processed (green) coffee beans (Coffea arabica L.). Int. J. Food. Microbiol. 85**,** 293-300.

Battilani, P., Pietri, A., Bertuzzi, A., Languasco, L., Giorni, P., Kozakiewicz, Z., 2003. Occurrence of ochratoxin A-producing fungi in grapes grown in Italy. J. Food. Prot. 66**,** 633-666.

Bayman, P., Baker, J.L., Doster, M.A., Michaillides, T.J., Mahony, N.E., 2002. Ochratoxin production by the *Aspergillus ochraceus* group and *Aspergillus alliaceus*. Appl. Environ. Microbiol. 68, 2326-2329.

Bingle, L. E., Simpson, T.J., Lazarus, C.M., 1999. Ketosynthase domain probes identify two subclasses of fungal polyketide synthase genes. Fungal. Genet. Biol. 26, 209-223.

Birch, A. J., Donovan, F. W., 1953. Studies in relation to Biosynthesis. I. Some possible routes to derivatives of Orcinol and Phloroglucinol. Aust. J. Chem. 6**,** 360-368.

Bridge, P. D., Arora, D.K., Reddy, C.A., Elander, R.P., 1998. Applications of PCR in mycology. CAB International. UK.

Bu'Lock, J. D., Sheperd, D., Winstanley, D.J., 1968. Regulation of 6-methylsalicylate and patulin synthesis in *Penicillium urticae*. Can. J. Microbiol. 15**,** 279 - 285.

Calvo, A. M., Gardner, H.W., Keller, N.P., 2001. Genetic connection between fatty acid metabolism and sporulation in *Aspergillus nidulans*. J. Biol. Chem. 276, 20766-20774.

Calvo, A. M., Wilson, R.A., Bok, J.W., Keller, N.P., 2002. Relationship between Secondary Metabolism and Fungal Development. Microbiol. Mol. Biol. Rev. 66, 447-459.

Cullen, D., Leong, S.A., Wilson, L.J., Henner, D.J., 1987. Transformation of *Aspergillus nidulans* with the hygromycin-resistance gene, *hph*. Gene 57, 21-26.

Dalcero, A., Magnoli, C., Hallak, C., Chiacchiera, S.M., Palacio, G., Rosa, C.A., 2002. Detection of ochratoxin A in animal feeds and capacity to produce this mycotoxin by *Aspergillus* section Nigri in Argentina. Food. Addit. Contam. 19, 1065-1072.

Dao, H.P., Mathieu, F., Lebrihi, A., 2005. Two primer pairs to detect OTA producers by PCR method. Int. J. Food Microbiol. 104**,** 61-67.

Färber, P., Geisen, R., 2004. Analysis of Differentially-Expressed Ochratoxin A Biosynthesis Genes of *Penicillium nordicum*. Eur. J. Plant Pathol. 110, 661-669.

Frisvad, J. C., Frank, J.M., Houbraken, J.A.M.P., Kuijpers, A.F.A., Samson, R.A., 2004. New ochratoxin A producing species of *Aspergillus section Circumdati*. Stud. Mycol. 50, 23–43.

Fujii, I., Watanabe, A., Sankawa, U., Ebizuka, Y., 2001. Identification of Claisen cyclase domain in fungal polyketide synthase WA, a naphthopyrone synthase of *Aspergillus nidulans*. Chem. Biol. 8, 189-197.

Gardes, M., Bruns, T. D., 1993. TS primers with enhanced specificity for Basidiomycetes-applicatin to the identification of mycorrhizae and rusts. Mol. Ecol. 2, 113-118.

Gaucher, G.M., Shepherd, M.G., 1968. Isolation of orsellinic acid synthase. Biochem. and Biophys. Res. Com. 32, 664-671.

Graziani, S., Vasnier, C., Daboussi, M.J., 2004. Novel Polyketide Synthase from *Nectria haematococca*. Appl. Environ. Microbiol. 70, 2984–2988.

Harris, J.P., Mantle, P.G., 2001. Biosynthesis of ochratoxins by *Aspergillus ochraceus*. Phytochemistry 58, 709-716.

Hendrickson, L., Davis, C.R., Roach, C., Nguyen, D.K., Aldrich, T., McAda, P.C., Reeves, C.D., 1999. Lovastatin biosynthesis in *Aspergillus terreus*: characterization of blocked mutants, enzyme activities and a multifunctional polyketide synthase gene. Chem. Biol. 6, 429-439.

Hopwood, D.A., 1988. Towards an understanding of gene switching in *Streptomyces*, the basis of sporulation and antibiotic production. T. Proc. R. Soc. Lond. 235, 121-138.

Huff, E.E., Hamilton, P., 1979. Mycotoxin-their biosynthesis in fungi: ochratoxin metabolites of combined pathways. J. Food. Port. 42, 815-820.

Jorgensen, K., 1998. A survey of pork, poultry, coffee, beer and pulses for Ochratoxin A. Food Addit. Contam. 15, 550-554.

Jorgensen, K., Jacobsen, J. S., 2002. Occurrence of ochratoxin A in Danish wheat and rye, 1992-1999. Food Addit. Contam. 19, 1184-1189.

Kao, C.M., Katz, L., Khosla, C., 1994. Engineered biosynthesis of a complete macrolactone in a heterologous host. Science 265, 509-512.

Karolewiez, A., Geisen, R., 2005. Cloning a part of the ochratoxin A biosynthetic gene cluster of *Penicillium nordicum* and characterisation of the ochratoxin polyketide synthase gene. Syst. Appl. Microbiol. 28**,** 588-595.

Kennedy, J., Auclair, K., Kendrew, S.G., Park, C., Vederas, J.C., Hutchinson, C.R., 1999. Modulation of polyketide synthase activity by accessory proteins during lovastatin biosynthesis. Science 284**,** 1368-1372.

Kim, Y. T., Lee, Y.R., Jin, J., Han, K.H., Kim, H., Kim, J.C., Lee, T., Yun, S.H., Lee, Y.W., 2005. Two different polyketide synthase genes are required for synthesis of zearalenone in *Gibberella zea*. Mol. Microbiol. 58, 1102-1113.

Kuiper-Goodman, T., Scott, P. M., 1989. Review: risk assessment of the mycotoxin ochratoxin A. Biomed. Environ. Sci. 2, 179-248.

Lee, T., Yun, S.H., Hodge, K.T., Humber, R.A., Krasnoff, S.B., Turgeon, G.B., Yoder, O.C., Gibson, D.M., 2001. Polyketide synthase genes in insect- and nematode-associated fungi. Appl. Microbiol. Biotechnol. 56**,** 181-187.

Liou, G.F., Khosla, C., 2003. Building-block selectivity of polyketide synthases. Curr. Opin. Chem. Biol. 7, 279-284.

Lui, D., Caloe, C., Biard, R., Pedersen, J., 2000. Rapid mini preparation of fungal DNA for PCR. J. Clin. Microboil. 38**,** 471.

Magan, N., Olsen, M., 2004. Mycotoxins in Food: Detection and Control. Cambridge: DC/Woodhead Publishing Limited.

Metz, J. G., Roessler, P., Facciotti, D., Levering, C., Dittrich, F., Lassner, M., Valentine, R., Lardizabal, K., Domergue, F., Yamada, A., Yazawa, K., Knauf, V., Brows, J., 2001. Production of polyunsaturated fatty acids by polyketide synthase in both prokaryotes and eukaryotes. Science 293, 290-293.

Nicholson, T. P., Rudd, B.A.M., Dawson, M., Lazarus, C.M., Simpson, T.J., Cox, R.J., 2001. Design and utility of oligonucleotide gene probes for fungal polyketide synthases. Chem. Biol. 8, 157-178.

O'Callaghan, J., J., Caddick, M.X., Dobson, A.D.W., 2003. A polyketide synthase gene required for ochratoxin A biosynthesis in *Aspergillus ochraceus*. Microbiol. 149, 3485-3491.

Petzinger, E., Ziegler, K., 2000. Ochratoxin A from a toxicological perspective. J. Vet. Pharmacol. Ther. 23, 91-98.

Sambrook, J., Fritsch, E.F., Maniatis , T., (Eds.), 1989. Molecular cloning: a laboratory Manual. Cold Spring Harbor laboratory press, Cold Spring Harbor.

Schmidt-Heydt, M., M., Richter, W., Michulec, M., Buttinger, G., Geisen, R., 2008. Comprehensive molecular system to study the presence, growth and ochratoxin A biosynthesis of *Penicillium verrucosum* in wheat. Food. Addit. Contam. 25, 989-996.

Southern, E.M., 1975. Detection of specific sequences among DNA fragments separated by get electrophoresis. J. Mol. Biol. 98**,** 503-517.

Tang, C. M., Cohen, J., Holden, D.W., 1992. An *Aspergillus fumigatus* alkaline protease mutant constructed by gene disruption is deficient in extracellular elastase activity. Molecular. Microbiol. 6, 1663-1671.

Teren, J., Varga, J., Hamari, Z., Rinyu, E., Kevei, F., 1996. Immunochemical detection of ochratoxin A in black *Aspergillus* strains. Mycopathologia 134, 171-176.

Varga, J., Rigo, K., Kocsube, S., Farkas, B., Pal, K., 2003. Diversity of polyketide synthase gene sequences in *Aspergillus* species. Res. Microbiol. 154, 593-600.

Yu, J. H., Leonard, T. J., 1995. Sterigmatocystin biosynthesis in *Aspergillus nidulans* requires a novel type I polyketide synthase. J. Bacteriol. 177, 4792-4800.

Zimmerli, B., Dick, R., 1996. Ochratoxin A in table wine and grape-juice: Occurrence and risk assessment. Food. Addit. Contam. 13**,** 655-668.

Table 1. Primers used in this study

Primer name	Sequence (5'-3')
ATR1	YTG5GC5CCYTG5CC5GTDAA
ATR2	CATRTGRTGIGARTGRTAIGC
AoKS1	CGGAAGGCCGGCCTAGATCCAGCC
AoKS1F	GAAGCCGTCGAGGCCGCCGGTCTG
AoKS1R	CAATGCGAATTGCCTCTATTTC
AoKSF	CGGCCAATCTGGGAGATTTGGC
*hph*2F	CGGGGGCAATGAGATATGAAAAAG
*hph*2R	GAACCCGCTCGTCTGGCTAAG
LC12F	CTATGACTTACGCGGGACAAG
LC12R	AAGGCAGATACAATGGCCTGC
TubF	CTCGAGCGTATGAACGTCTAC
TubR	AAACCCTGGAGGCAGTCGC

Figure legends and Figures

Fig. 1.

Chemical structures of ochratoxin A (a) and mellein (b).

Fig. 2.

Alignment of the deduced amino acid sequence of *A. westerdijkiae aoks1* with other PKSs: *lovB* (Accession N°: AAD39830, lovastatin nonaketide synthase in *A. terreus*); *mlcA* (Accession N°:BAC20564, compactin nonaketide synthase in *P. citrinum*); *otapksPN* gene (Accession N°:AY557343, OTA biosynthesis gene in *P. nordicum*); *aolc35-12* (Accession N°:AY583208) and *pks* (Accession N°:AY272043, OTA biosynthesis gene characterized by O'Callaghan et al. (2003)) in *A. ochraceus*. Red box indicates KS domain while green box indicates AT domain. Flash arrows indicate positions of different primers used in *extanding* aoks1 gene.

Fig. 3.

Schematic representation of transformation vector formation and *aoks1* gene disruption. (a) Using primer pair AoKS1F/AoKS1R (table 1), 1690 bp *aoks1* fragment containing *Sal*1 restriction site (indicated by triangle) was amplified from a 2 kb *aoks1* gene. PCR product was cloned into pCR2.1-Topo plasmid to generate plasmid TopoKS. pID2.1 plasmid vector was restricted with *Sal*1 (indicated by triangle) to obtain *hph* cassette (2.4 kb). TopoKS was restricted with *Sal*1 and ligated with *hph* cassette to genarate TopoKS*hph* transformation vector. Different colors on the *aoks1* gene indicate different functional domains i.e. β-ketoacyl synthase (KS) and acyltransferase (AT). (b) Protoplasts of *A. westerdijkiae* was prepared and *aoks1* gene was disrupted using TopoKS*hph* vector to obtain *aoΔks1* mutant. The small red lines below *aoΔks1* indicate position of radioactively labelled probes i.e. KP: *aoks* gene specific probe amplified by using primer pair AoKSF / AoKS1R and, HP: *hph* gene specific probe amplified by using primer pair *hph*2F /*hph*2R.

Fig. 4.

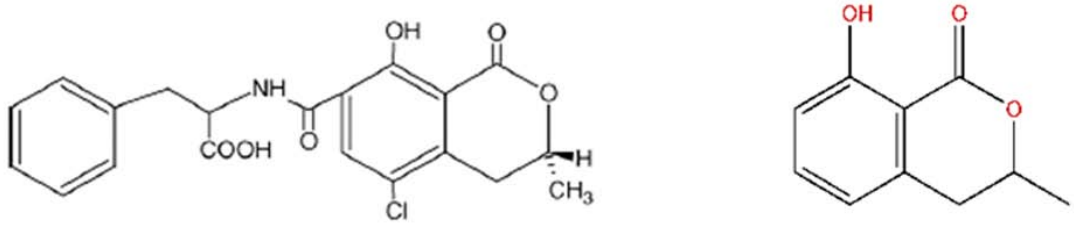
Kinetic production of secondary metabolites and comparison of two PKS genes expression, in *A. westerdijkiae* NRRL 3174. (a) Kinetic production of mellein and OTA in a growing culture of *A. westerdijkiae* NRRL 3174 at 25°C in synthetic medium during a time course of 2-18 days. (b) Profile of *aoks1* gene expression (upper panel) and *aolc35-12* gene expression (middle panel) by RT-PCR. Beta tubulin was used as loading control (bottom panel).

Fig. 5.

PCR screening and southern blot hybridization of the transformants. (a) the PCR product of 0.6 kb (lane 1) obtained with primers *hph*2F/*hph*2R and the 1.2 kb product (lane 4) obtained with AoKS1F/*hph*2R confirmed the integration of the *hph* gene cassette into the genome of *A. westerdijkiae* NRRL 3174 (*aoΔks1* transformants). No product was amplified in *A. westerdijkiae* wild-type with *hph*2F/*hph*2R (lane 2) and AoKS1F/*hph*2R (lane3). Lane M: size markers (Promega). (b) Southern blot screening of transformants: Genomic DNA of wild type *A. westerdijkiae* and *ao*Δ*ks1* transformants were extracted and digested with *Bts*1 restriction enzyme (indicated by triangle). The products were analyzed through electrophoresis and then transferred to nylon membrane. Probing of the membrane with radioactively labelled *hph* fragment (HP) resulted into; lane 1: wild type *A. westerdijkiae* and lane 2: *ao*Δ*ks1* mutant. Probing of the membrane with radioactively labelled *aoks1* fragment (KP) resulted into; lane 3: wild type *A. westerdijkiae* and lane 4: *ao*Δ*ks1* mutant.

Fig. 6.

Tracking secondary metabolites production in wild type *A. westerdijkiae* NRRL 3174 and *ao*Δ*ks1* mutant. (a) Characteristic fluorescence of OTA under UV light observed in wild type *A. westerdijkiae* NRRL 3174 CYA culture, and the absence of fluorescence in *ao*Δ*ks1* mutant culture. (b) HPLC traces and UV spectra of mellein (eluted at 27 min) and OTA (eluted at 34 min) in wild type *A. westerdijkiae* NRRL 3174 (black line) and *ao*Δ*ks1* mutant (pink colour line). (c) Kinetic production of mellein in *ao*Δ*ks1* mutant during a time course of 2 to 18 days.



a b

Figure 1

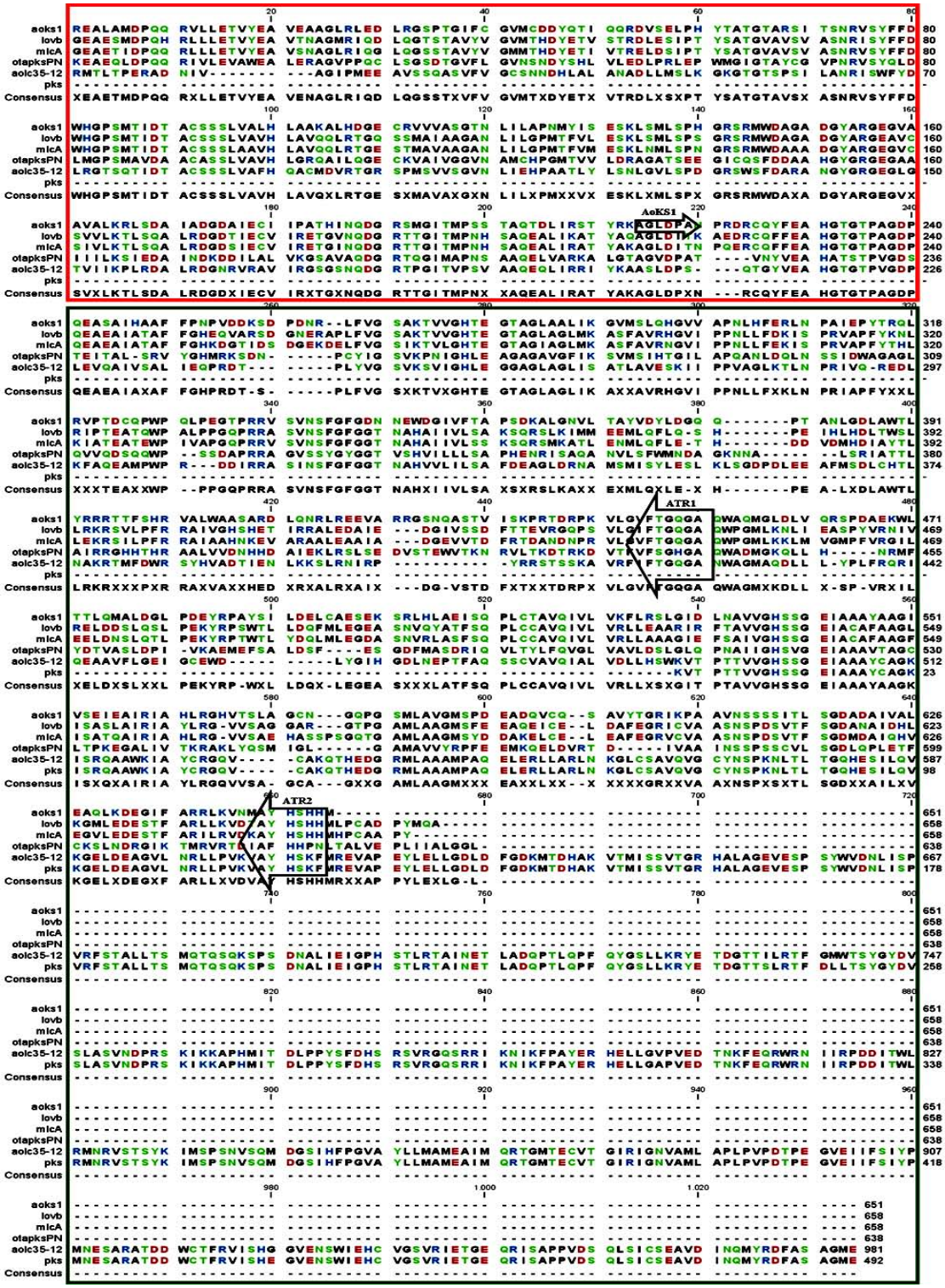


Figure 2

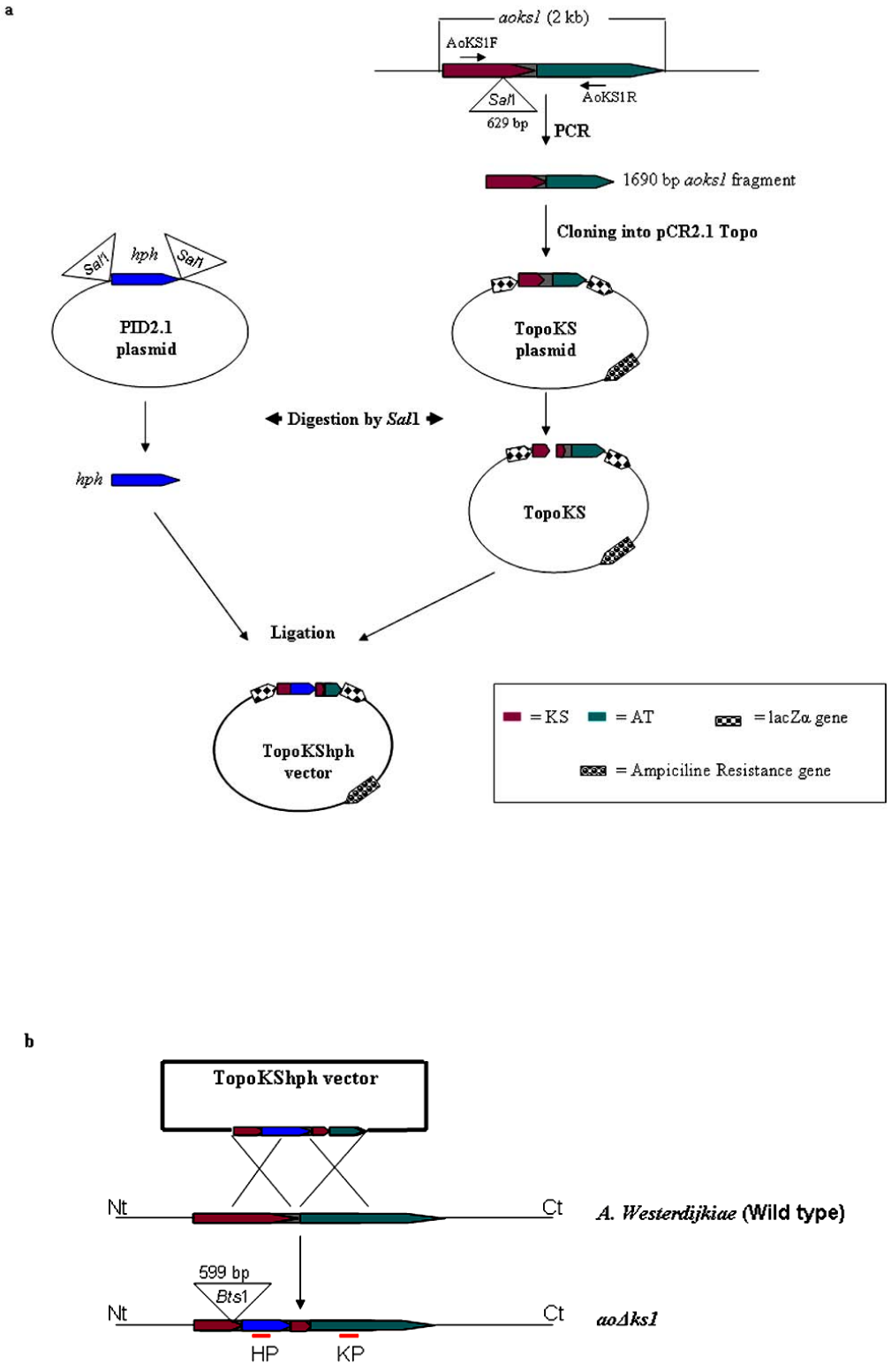
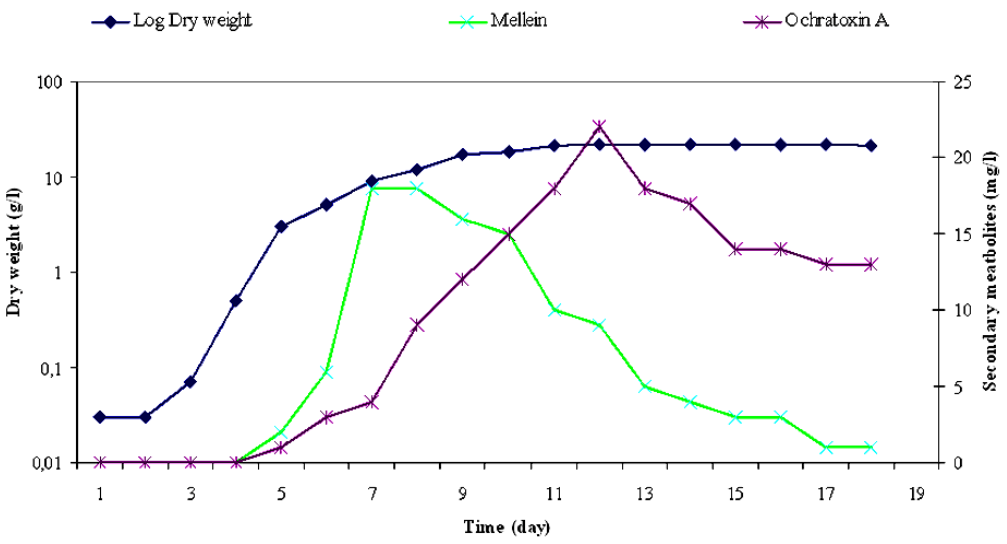

a
aoks1 (2 kb)
AoKS1F
AoKS1R
SalI
629 bp
PCR
1690 bp aoks1 fragment
Cloning into pCR2.1 Topo
SalI
hph
SalI
PID2.1 plasmid
TopoKS plasmid
Digestion by SalI
hph
TopoKS
Ligation
TopoKShph vector
= KS
= AT
= lacZα gene
= Ampiciline Resistance gene
b
TopoKShph vector
Nt
Ct
A. Westerdijkiae (Wild type)
599 bp
Bts1
Nt
Ct
aoΔks1
HP
KP

Figure 3

a


Log Dry weight
Mellein
Ochratoxin A
100
10
1
0,1
0,01
Dry weight (g/l)
25
20
15
10
5
0
Secondary metabolites (mg/l)
1
3
5
7
9
11
13
15
17
19
Time (day)

b

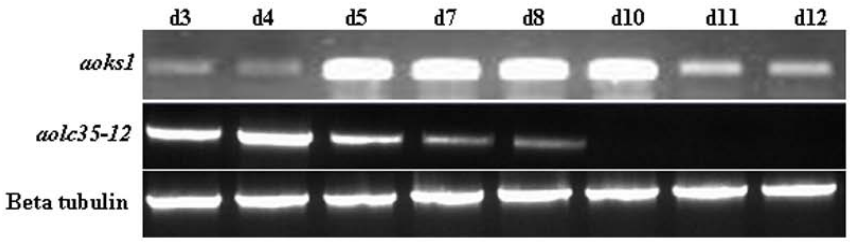

d3
d4
d5
d7
d8
d10
d11
d12
aoks1
aolc35-12
Beta tubulin

Figure 4

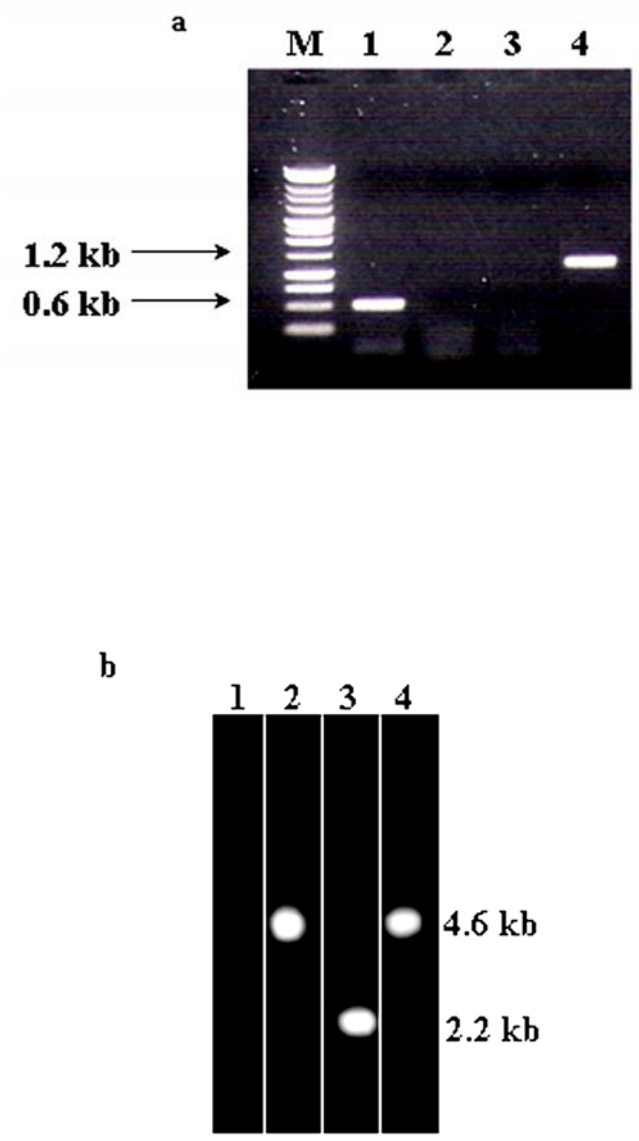
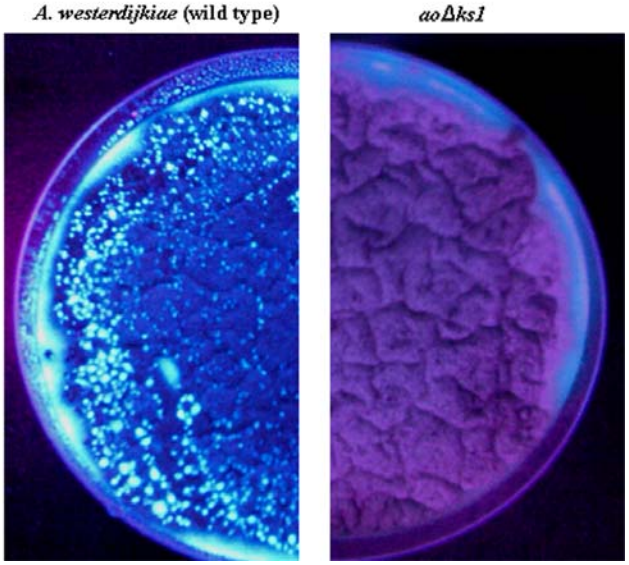
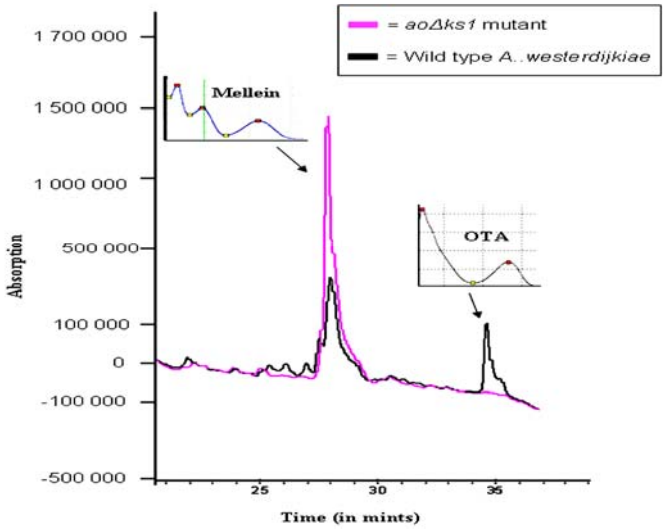


Figure 5

a


A. westerdijkiae (wild type)
aoΔks1

b


= aoΔks1 mutant
= Wild type A..westerdijkiae
Mellein
OTA
1 700 000
1 500 000
1 000 000
500 000
100 000
0
-100 000
-500 000
Absorption
25
30
35
Time (in mints)

c

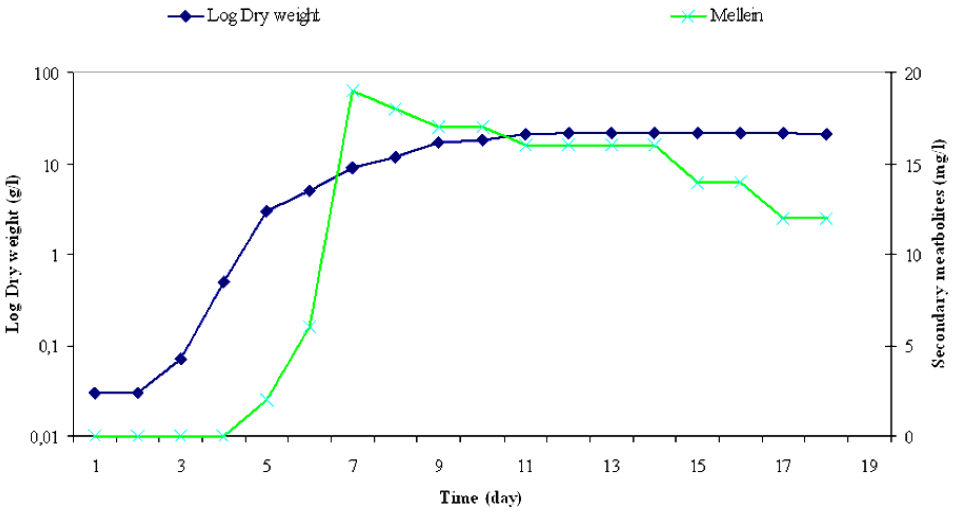

Log Dry weight
Mellein
100
10
1
0,1
0,01
20
15
10
5
0
Log Dry weight (g/l)
Secondary meatbolites (mg/l)
1
3
5
7
9
11
13
15
17
19
Time (day)

Figure 6

Chapter II

Article 2: In Preparation for World Mycotoxin Journal

3.2 A polyketide synthase gene "*aolc35-12*" controls the differential expression of ochratoxin A gene "*aoks1*" in *Aspergillus westerdijkiae*

Nafees Bacha[(1)], Florence Mathieu[(1)], Thierry Liboz[(1)] and Ahmed Lebrihi[(1)**]

[1] Département Bioprocédés et Systèmes Microbiens, Laboratoire de Génie Chimique UMR5503 (CNRS/INPT/UPS), Ecole Nationale Supérieure Agronomique de Toulouse, Institut National Polytechnique de Toulouse, 1, avenue de l'Agrobiopôle, BP32607, 31326 Castanet Tolosan, France.

[**] Corresponding author

Tel: 00 33 (0)5 62 19 39 44

Fax: 00 33 (0)5 62 19 39 01

E-mail: lebrihi@ensat.fr

Abstract

Ochratoxine A (OTA) is a possible human carcinogen produced by several species of *Aspergillus* and *Penicillium* including *Aspergillus westerdijkiae*. A putative polyketide synthase gene "*aolc35-12*" has been partially cloned in *A. westerdijkiae* by using genome walking approach. The predicted amino acid sequence of the 3.22 kb clone was found to be an arthologue (> 97 % similar) of the previously identified OTA-pks gene from *A. ochraceus* (O'callaghan *et al*., 2003). *aolc35-12* further displayed similarity of 60 % to a hypothetical PKS gene corresponding to OTA cluster in *A. niger* while 34-40 % similarities with other pks genes in *Penicillium marneffei, A. clavatus, A. terreus,* and *A. fumigatus*. The OTA negative mutant "aoΔlc35-12" was generated by inactivation of *aolc35-12* gene through the insertion of *Escherichia coli* hygromycin B phosphotransferase gene cassette. The mutation produced no impact on the biosynthesis of mellein which was previously proposed as intermediate of OTA. Further, study of the differential expression of *aolc35-12* and another OTA gene *aoks1* (Bacha *et* al., 2008) in the wild-type *A. westerdijkiae* and aoΔlc35-12 mutant revealed that *aolc35-12* is important for the expression of *aoks1* gene and hence for the biosynthesis of OTA.

Key Words: *Aspergillus westerdijkiae*, Polyketide synthase, Ochratoxin A biosynthesis, Mellein, *aolc35-12*.

1. Introduction

Ochratoxin A (OTA) is a toxic polyketide metabolite primarily produced by *Aspergillus,* but also produced by *Penicillium*. *A. westerdijkiae*, which was recently dismembered from *A. ochraceus* (Frisvad *et al.*, 2004a) is one of the most important *Aspergillus* species producing this metabolite (Atoui *et al.*, 2006; Balcells *et al.*, 1995; Gaucher & Shepherd, 1968; Torres *et al.*, 1998). Based on sufficient evidence in experimental animals, OTA is reasonably anticipated to be a human nephrotoxin which also displays hepatotoxic, teratogenic, and immunosuppressive properties. OTA has also been classified as a possible human carcinogen (category 2B), by the International Agency for Research on Cancer (Kuiper-Goodman & Scott, 1989; Petzinger & Ziegler, 2000).

Ochratoxin A is a natural contaminant on corn, peanuts, storage grains, cottonseed, and decaying vegetation (Budavari, 1989). It has been detected in moldy cereals including wheat, maize, rye, barley, and oats, with cereal-based products typically accounting for 50–80 % of the average consumer intake of the mycotoxin (Jorgensen & Jacobsen, 2002). OTA has also been reported in other plant products including coffee beans, spices, nuts, olives, grapes, beans, and figs (Batista *et al.*, 2003; Battilani *et al.*, 2003; Bayman *et al.*, 2002; Jorgensen, 1998). The presence of OTA has been detected in a range of beverages (Zimmerli & Dick, 1996) and has also been reported in body fluids and kidneys of animals and humans (Magan & Olsen, 2004).

Based on the structure of OTA a mechanistic biosynthetic pathway has been previously proposed, according to which the heterocyclic portion of OTA is structurally similar to mellein (Huff & Hamilton, 1979). This authors proposed that mellein could be a precursor of OTA. In another experiment based on chemical complementation of labelled precursors of OTA, conclusions has been drawn that mellein does not seem to play a role in OTA biosynthesis (Harris & Mantle, 2001). This has been further confirmed when the OTA negative mutants in *A. westerdijkiae* produced mellein (Bacha *et al.*, 2009a).

Although polyketide secondary metabolite varies remarkably in their structures and function, their biosynthetic pathways apparently follow a common basic reaction scheme.

The key chain-building step of this reaction scheme is a decarboxylative condensation analogous to the chain elongation step of classical fatty acid biosynthesis (Birch & Donovan, 1953; Kao *et al.*, 1994). During this reaction the successive condensation step of small carbon precursor acid is catalyzed by a group of multifunction enzymes called polyketide synthases (PKSs), (Metz *et al.*, 2001). A typical fungal PKS is composed of modules known as domains e.g. ketosynthetase (KS), acyltransferase (AT) acyl carrier protein (ACP), dehydratase (DH), enoyl reductase (ER), ketoreductase (KR) and thioesterase (TE) etc (Graziani *et al.*, 2004).

The study of genetic nature of polyketide secondary metabolites have been facilitated by the introduction of degenerated primers based PCR cloning techniques (Bridge *et al.*, 1998). For this purpose, mainly the KS and AT domains i.e. the most conserved domains among different PKSs, have been targetted (Bingle *et al.*, 1999; Nicholson *et al.*, 2001). Based on the degenerated primers based techniques several genomes of filamentous fungi have been sequenced. The sequencing data revealed a large number of putative pks genes existing in filamentous fungi (Machida *et al.*, 2005; Yu *et al.*, 2005). By using the degenerated based PCR technique an OTA-PKS gene i.e. *aoks1* has been recently identified and characterized in *A. westerdijkiae* (Bacha *et al.*, 2009a). Similarly another OTA gene has been cloned in *A. ochraceus* (O'Callaghan *et al.*, 2003). One of the PKS gene identified in *A. carbonarius* was found to be differentially expressed during OTA producing conditions (Antonia *et al.*, 2008).

In this study, we report cloning and functional characterisation of a PKS gene "*aolc35-12*" required for the differential expression of OTA-PKS gene "*aoks1*" in *A. westerdijkiae*.

2. Materials and Methods

2.1. Fungal strain and culture conditions

A. westerdijkiae NRRL 3174 strain was grown for sporulation at 25°C on potato dextrose agar for 7 days. Spores were collected using a solution of 0.01% (v/v) Tween 80, counted by using Thoma Bright line counting chamber, 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 synthetic medium (SAM) at 25 °C for 2 to 18 days, without shaking. The composition of SAM (per liter of distilled water) was: 3 g NH_4NO_3, 26 g K_2HPO_4, 1 g KCl, 1 g $MgSO_4.7H_2O$, 10 mL mineral solution (composition per liter of distilled water: 70 mg $Na_2B_4O_7.10H_2O$, 50 mg $(NH_4)_6$ $Mo_7O_{24}.4H_2O$, 1000 mg $FeSO_4.7H_2O$, 30 mg $CuSO_4.5H_2O$, 11 mg $MnSO_4.H_2O$, 1760 mg $ZnSO_4.7H_2O$), and 50 g glucose. The pH of the medium was adjusted to 6.5 by the addition of 2 N HCl. Mycelium was harvested by filtration through a 0.45µM filter, grounded in liquid nitrogen and then stored at -80 °C before nucleic acid extraction. Three replications of each sample were analyzed.

2.2. Nucleic acid extraction

Rapid method of genomic DNA extraction (Lui *et al.*, 2000) was used for transformants screening by PCR. Large quantity genomic DNA was extracted by CTAB extraction method (Gardes & Bruns, 1993). The quality and quantity of DNA were estimated by measuring OD 260 nm / OD 280 nm and OD 260 nm respectively.

Total RNA was extracted from *A. westerdijkiae* using the Tri-reagent (Euromedex France) DNA / RNA/ Protein extraction kit. The quality and quantity of RNA was checked by the OD 260 nm / OD 280 nm ratio and agarose gel electrophoresis according to standard protocols (Sambrook *et al.*, 1989).

2.3. Cloning of *aolc35-12* gene in *A. westerdijkiae*

The previously cloned *aolc35-12* gene fragment (Atoui *et al.*, 2006) was extended by using degenerated primers based genome walking approach. The degenerated primers AT1, ATR and ATR1 were designed at the conserved regions **FTGQGAQ, AYHSSHM** and **YRDFASAG** respectively of different fungal PKSs present in the database (table 1

and Fig. 1). In a series of PCRs, the primers combinations LCF1 / AT1, LCF2 / ATR and LCF3 / ATR1 and LCF4 / LC12R2 were used to amplify various fragments of *aolc35-12* gene. Each individual PCR product was cloned in pCR2.1 Topo vector (Invitrogen) and sequenced. Alignment of the sequenced fragments was performed to search for consensus.

2.4. PCR reaction and sequencing

PCR was performed with the *Taq* recombinant polymerase (Invitrogen, USA). Amplification was carried out in a 50 µl reaction mixture containing: 5 µl of *Taq* polymerase 10× buffer, 1·5 µl of 50 mM $MgCl_2$, 1 µl of dNTP 10 mM of each (Promega), 1 µM of each primer, 1·5 U of *Taq*, about 200 ng of DNA genomic, H_2O up to 50 µl. Reaction conditions were: 94 °C for 4 min, (94 °C for 45 s, 53 °C for 45 s and 72 °C for 1 min) × 30 cycles followed by an incubation at 72 °C for 10 min. The amplified products were examined by 1 % (w/v) agarose (Promega) gel. The PCR products were cloned into pCR2.1-Topo vector according to the supplier's instructions. Sequencing of the fragments was performed by Genomexpress (Grenoble, France).

2.5. 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 CLC Main WorkBench (CLC Bio, Denmark)

2.6. Reverse transcription PCR reaction

The expression of *aolc35-12* in *A. westerdijkiae* was examined by using reverse transcription PCR (RT-PCR) with specific pairs of primers Aolc12F / Aolc12R designed from the KS domain of *aolc35-12* (Table 1). For RT-PCR, total RNA was treated with DNase I (Promega) to remove DNA contamination. cDNA was synthesized from each sample with Advantage RT- for-PCR Kit (BD Biosciences) according to the supplier's manual. cDNA amplifications were performed using Taq recombinant polymerase

(Invitrogen,USA). Beta tubulin was used as positive control using primers TubF and TubR (Table 1).

2.7. Disruption of *aolc35-12* gene in *A. westerdijkiae*

Escherichia coli hygromycin B phosphotransferase gene (*hph*) flanked by *A. nidulans trpC* promoter and terminator sequences (Cullen *et al.*, 1987) from plasmid pID2.1 (Tang *et al.*, 1992) was used to disrupt *aolc35-12* gene in the genome of *A. westerdijkiae*. A modified form of PCR- mediated generation of gene disruption technique (Kuwayama *et al.*, 2002) was used to construct a *hph* cassette containing transformation vector (Fig. 2a). This technique is composed of three rounds of PCRs; in first round the full length *hph* cassette, 5`aolc35-12 containing *hph* tail and 3`aolc35-12 containing *hph* tail is amplified; in second round the 5` aolc35-12 containing *hph* tail fragment is fused with the full length *hph* cassette to obtained *hph* cassette flanked by 5` aolc35-12 fragment; and in the third round *hph* cassette flanked by 5` aolc35-12 fragment was fused with 3` aolc35-12 containing *hph* tail fragment to obtained *hph* cassette flanked at the 5`and 3` by aolc35-12 fragments. Different primers used during this approach are listed in table 1. The final fusion PCR product was cloned into pCR2.1-Topo plasmid (Invitrogen) generating the TopoLC12*hph* plasmid.

To construct the complementation vector Comp-lc12, PCR primers LC12fullF and LC12fullR were designed to amplify a 3 kb *aolc35-12* gene fragment. The amplified fragment was cloned in pCR2.1-Topo plasmid.

The *aolc35-12* gene inactivation and complementary mutant production were achieved by preparing protoplasts of the corresponding strains and transforming with TopoMS*hph* and Comp-lc12 respectively as previously described (Jaoanne *et al.*, 2007; O'Callaghan *et al.*, 2003) (Fig. 2b). 40 mg/mL lysing enzyme (Sigma) was used for the preparation of protoplasts.

2.8. Screening of the transformants

The *aolc35-12* disrupted mutants "*aoΔlc35-12*" and genetically complemented mutants *ao+lc35-12* were initially selected on YES medium (20 g/l of yeast extract, 1 M sucrose)

supplemented with 150 µg/mL of hygromycin B. Transformant plates were incubated at room temperature for 24 h and then transferred to 30°C for 4 days. The positively screened transformants were further screened through PCR, using *hph* gene specific primers *hph*2F and *hph*2R and *aolc35-12* gene specific primer LC12fullF (Table 1). Finally the disruption was confirmed through southern hybridization (Southern, 1975), in which total genomic DNA from both wild type *A. westerdijkiae ao*Δ*lc35-12* and *ao+lc35-12* transformants were digested with restriction enzyme *HindIII*. This restriction enzyme was chosen because it cut only once in the 5´- flanking region of *ao*Δ*lc35-12* transformant (position 1171 bp) but does not cut the *hph* cassette or 3´- flanking end of the *ao*Δ*lc35-12* (Fig. 2b). The digested DNA was then transferred to nylon membrane (Amersham, France) and probed with radioactively labelled DNA fragments of *aolc35-12* gene "lc12p", amplified through primer pair SotLCF / LC12fullR (probes position is shown in Fig. 3b).

2.9. Fluoresence study

In order to check the loss of OTA production by the *ao*Δ*lc35-12* mutant, Czapek Yeast extract Agar (CYA) plates of both wild type *A. westerdijkiae* and *ao*Δ*lc35-12* mutant were incubated for 10 days at 25°C and then illuminated with UV light (265 nm). Production of OTA is characterized by a blue fluorescent halo under UV.

2.10. Extraction of secondary metabolites

For secondary metabolites extraction, 30 mL filtrate sample of the culture medium was acidified with 200 µL of 12 N HCl, mixed with 30 mL chloroform and vigorously shaken for 10 min. The solvent phase was then decanted, dried under vacuum and re-dissolved in 0.5 mL Methanol. 20 µL of the sample was then further analysed by HPLC.

2.11. High-performance liquid-chromatography (HPLC) analysis

The HPLC apparatus consisted of a solvent delivery system, with both fluorescence (λ_{ex} = 332 nm; λ_{em} = 466 nm) and UV detectors (BIO-TEK, Milan, Italy). The analytical column used was a 150 x 4.6 mm Uptisphere 5 µm C18 ODB fitted with a guard column of 10 x 4 mm. The column temperature was 30 °C. Kroma 3000 (BIO-TEK) was the data

acquisition system. Injections were done with an auto-injector (BIO-TEK, Milan, Italy) and the injection volume was 20 µL. The samples were analyzed by linear gradient elution using 0.2 % glacial acetic acid in 99.8 % water (v/v) (A) and 100 % acetonitrile (HPLC grade) (B). The crude extract was analyzed using a linear elution gradient over 45 min at a flow rate of 1 ml/min, starting from 10 to 50% solvent B over the first 30 min, continued by a linear gradient to 90 % of B in 5 min, followed by an isocratic flow of 90% solvent B for 8 min, and a return to initial conditions over the last 2 min of the run.

Secondary metabolites of *A. westerdijkiae* NRRL 3174 were detected by comparing the elution time and maximum absorption of UV with the standards (Sigma Aldrich France). All the standards were used at a concentration of 10µg/mL. Mellein (λ_{max} = 214; 245 and 314 nm) and ochratoxin A (λ_{max} = 216; 250 and 332 nm) were released at 27 min and 34 min respectively.

2.12. Sequence accession number

The sequence obtained was deposited in Genbank under the accession number AY583208.

3. Results and discussion

3.1. Sequencing and characterization of *aolc35-12* gene in *A. westerdijkiae*

Polyketide secondary metabolites are the vital group of naturally accruing polycetates with more then 10,000 biologically active compounds. Several genes coding the polyketide catalysing enzymes "polyketide synthases (PKSs)" have been identified in various organisms (Bingle *et al.*, 1999; Lee *et al.*, 2001b; Varga *et al.*, 2003). Recently in our laboratory a 700 bp ketosynthase (KS) domain of a PKS gene *aolc35-12* has been identified in *A. westerdijkiae* by using the degenerated primers of Bingle *et al*., (1999) (Atoui *et al.*, 2006). To functionally characterize *aolc35-12,* we have prolonged the identified KS domain to a length of 3.233 kb through degenerated primers based genome walking approach (Figure 1). We have utilized similar technique to clone and characterize another PKS gene *aoks1* correspond to OTA biosynthesis in *A. westerdijkiae* (Bacha *et al.*, 2009a).

Homology search revealed that amino acid sequence of *aolc35-12* is arthologous (98 % similar) to the OTA-PKS gene identified in *A. ochraceus* (O'Callaghan *et al.*, 2003). The gene also displayed similarities of 60 % to another PKS gene corresponding to an OTA cluster in *A. niger* (Pel *et al.*, 2007) and only 38 % to *aoks1* gene in *A. westerdijkiae* (Bacha *et al.*, 2009a). Further similarities of 38-40 % were observed between *aolc35-12* and various pks gene in *Penicillium marneffei, A. clavatus, A. terreus* and *A. fumigatus* (Fig. 1). Based on the high degree similarities between *aolc35-12* and various OTA-PKS genes, we assumed that *aolc35-12* could be involved in OTA biosynthesis in *A. westerdijkiae*.

3.2. Functional characterization of *aolc35-12* gene in *A. westerdijkiae*

By using TopoLC12*hph* in a protoplast based transformation technique, *aolc35-12* gene was inactivated in the genome of *A. westerdijkiae*. The transformants were subsequently passed through a series of confirmatory tests. After southern hybridization (Fig. 3a), two *aolc35-12* gene disrupted mutants i.e. *ao*Δ*lc35-12a and ao*Δ*lc35-12b,* one ectopic mutant i.e. *ectlc12* and wild-type *A. westerdijkiae* were grown on solid CYA medium. No difference was observed in their colony sizes, growth patterns, visual pigments and

conidial counts. In contrary it has been observed that *ao*Δ*lc35*-12 has lost the characteristic florescence of OTA (Fig. 3b). This character of OTA producing species has been used as an early test in order to identify OTA negative mutants (O'Callaghan *et al.*, 2003). Further filtrate of the 10 days old liquid culture of wild type *A. westerdijkiae*, the two *ao*Δ*lc35-12* mutants and *ectlc12* mutant were used to extract secondary metabolites. The HPLC profiles of the extracted secondary metabolites revealed that inactivation of *aolc35-12* gene by the insertion of *hph* cassette disrupted the production of OTA (Fig. 4), but produced no impact on the biosynthesis of mellein or hydroxymellein. Due to structural similarities with OTA, these two compounds were previously proposed as intermediate in the biosynthetic pathway of OTA (Huff & Hamilton, 1979). This hypothesis was initially ruled out by Harris and Mantle (2001), who used labbelled precursors of OTA and suggested that mellein has no role in the biosynthesis of OTA. Most recently during the characterization of another OTA gene in *A. westerdijkiae*, Bacha *et al*., 2009 demonstrated that the OTA negative mutants are still producing mellein and hydroxymellein. These observations strongly support our findings about the mellein independent biosynthesis of OTA in *A. westerdijkiae*. Our observations further suggest that *aolc35-12* could be the second OTA gene in *A. westerdijkiae*, arthologous to the OTA-PKS gene identified in *A. ochraceus* by O'Callaghan *et al.*, (2003). To date, only three cases have been reported that involve two different fungal PKSs essential for a single polyketide: a set of two unusual type I multifunctional PKSs for the biosynthesis of lovastatin and compactin in *A. terreus* and *P. citrinum* respectively (Abe *et al.*, 2002; Hendrickson *et al.*, 1999; Kennedy *et al.*, 1999) and two PKS have been reported to be involved in the biosysnthesis of the mycotoxins zearaleone in *Gibberella* zea (Kim *et al*., 2005) and T toxin in *Cochliobolus heterosphorus* (Baker *et al*., 2006).

3.3. Secondary metabolite production and expression of aolc35-12 in *A. westerdijkiae*

To study the enter-relation between the two OTA-PKS genes i.e. *aoks1* (Bacha *et al.*, 2009a) and *aolc35-12*, we followed their transcriptional signals during the growth of *ao*Δ*ks1* (Bacha *et al.*, 2009a) and *ao*Δ*lc35-12* mutants and wild-type *A. westerdijkiae*. Transcriptional signals of *aolc35-12* gene were observed from day 3 of wild-type *A. westerdijkiae* growth, reached a maximum at day 4 and then gradually decreased to

disappear after day 8 (Fig. 5). The expression pattern of this gene is totally different from the other OTA gene *aoks1*. We have recently demonstrated during the characterization of *aoks1* gene that transcriptional signals of this gene started at day 3, stayed constant at maximum level from day 5 to day 10 and then disappeared after day 12 (Bacha *et al.*, 2009a).

Interestingly we have observed that *aolc35-12* gene normally expressed in *ao*Δ*ks1* mutant, while the transcriptional signals of *aoks1* disappeared in *aolc35-12* mutant (Fig. 5). These observations suggest that *aolc35-12* gene differentially controls the expression of *aoks1* gene. In a similar experiment Antonia *et al.*, (2009) demonstrated that the OTA-PKS gene in *A. carbonarius* is differentially expressed in OTA permissive condition when compared with OTA suppressive condition. Hence these observations suggest that *aolc35-12* could code for certain polyketide compound important for the induction of OTA biosynthesis system in *A. westerdijkiae*.

Acknowledgements

The authors are grateful to the Higher Education Commission of Pakistan for according PhD scholarships to Mr. Nafees Bacha.

References

Abe, Y., Suzuki, T., Ono, C., Iwamoto, K., Hosobuchi, M. & Yoshikawa, H. (2002). Molecular cloning and characterization of an ML-236B (compactin) biosynthetic gene cluster in *Penicillium citrinum*. *Mol Genet Genomics* **267**, 636-646.

Antonia, G., Giancarlo, P., Michele, S., Filomena, E., Abdelhamid, A., Alan, D. W. D. & Giuseppina, M. (2008). Characterisation of a pks gene which is expressed during ochratoxin A production by *Aspergillus carbonarius*. *Int J Food Microbiol* **129**, 8-15.

Atoui, A., Dao., P., Mathieu, F. & Lebrihi, A. (2006). Amplification and diversity analysis of Ketosynthase domains of putative polyketide synthase gene in *Aspergillus ochraceus* and *Aspergillus carbonarius* producers of ochratoxin A. *Mol Nutr Food Res* **50**, 448-493.

Bacha, N., Atoui, A., Mathieu, F., Liboz, T. & Lebrihi, A. (2009). Aspergillus westerdijkiae polyketide synthase gene "aoks1" is involved in the biosynthesis of ochratoxin A. *Fungal Genetics and Biology* **46**, 77-84.

Baker, S. E., Kroken, S., Asvarak, T., Inderbitzin, P., Li, B. Y., Shi, L., Yoder, O. C. & Turgeon, B. G. (2006). Two polyketide synthase-encoding genes are required for biosynthesis of the polyketide virulence factor, T-toxin, by *Cochliobolus heterostrophus*. *Mol Plant Microbe Interact* **19**, 139-149.

Balcells, M., Canela, R., Coll, j., Sanchis, V. & Torres, M. (1995). Effect of fungal metabolites and some derivatives against *Tribolium castaneum* (Herbst) and *Nezara viridula* (L.). *Pestic sci* **45**, 319-323.

Batista, L. R., Chalfoun, S. M., Prado, G., Schwan, R. F. & Wheals, A. E. (2003). Toxigenic fungi associated with processed (green) coffee beans (Coffea arabica L.). *Int J Food Microbiol* **85**, 293-300.

Battilani, P., Pietri, A., Bertuzzi, A., Languasco, L., Giorni, P. & Kozakiewicz, Z. (2003). Occurrence of ochratoxin A-producing fungi in grapes grown in Italy. *J Food Prot* **66**, 633-666.

Bayman, P., Baker, J. L., Doster, M. A., Michaillides, T. J. & Mahony, N. E. (2002). Ochratoxin production by the *Aspergillus ochraceus* group and *Aspergillus alliaceus*. *Appl Environ Microbiol* **68**, 2326-2329.

Bingle, L. E., Simpson, T. J. & Lazarus, C. M. (1999). Ketosynthase domain probes identify two subclasses of fungal polyketide synthase genes. *Fungal Genet Biol* **26**, 209-223.

Birch, A. J. & Donovan, F. W. (1953). Studies in relation to Biosynthesis. I. Some possible routes to derivatives of Orcinol and Phloroglucinol. *Aust J Chem* **6**, 360-368.

Bridge, P. D., Arora, D. K., Reddy, C. A. & Elander, R. P. (1998). *Applications of PCR in mycology*: CAB International. UK.

Budavari, S. E. (1989). *The Merck Index*, 11 edn: Merck Co. Inc., Rahway, NJ.

Cullen, D., Leong, S. A., Wilson, L. J. & Henner, D. J. (1987). Transformation of *Aspergillus nidulans* with the hygromycin-resistance gene, *hph*. *Gene* **57**, 21-26.

Frisvad, J. C., Frank, J. M., Houbraken, J. A. M. P., Kuijpers, A. F. A. & Samson, R. A. (2004). New ochratoxin A producing species of *Aspergillus section Circumdati*. *Stud Mycol* **50**, 23–43.

Gardes, M. & Bruns, T. D. (1993). TS primers with enhanced specificity for Basidiomycetes-applicatin to the identification of mycorrhizae and rusts. *Mol Ecol* **2**, 113-118.

Gaucher, G. M. & Shepherd, M. G. (1968). Isolation of orsellinic acid synthase. *Biochem and Biophys Res Com* **32**, 664-671.

Graziani, S., Vasnier, C. & Daboussi, M.-J. (2004). Novel Polyketide Synthase from *Nectria haematococca*. *Appl Environ Microbiol* **70**, 2984–2988.

Hendrickson, L., Davis, C. R., Roach, C., Nguyen, D. K., Aldrich, T., McAda, P. C. & Reeves, C. D. (1999). Lovastatin biosynthesis in *Aspergillus terreus*: characterization of blocked mutants, enzyme activities and a multifunctional polyketide synthase gene. *Chem Biol* **6**, 429-439.

Huff, E. E. & Hamilton, P. (1979). Mycotoxin-their biosynthesis in fungi: ochratoxin metabolites of combined pathways. *J Food Port* **42**, 815-820.

Jaoanne, W. S. H., Corentin, H., Nafees, B., Richard, O. C., Claude, L., Giséle, B., Michel, R., Pierre, R. & Bernard, D. (2007). Regulation and role of the STE12-like transcription factor from the plant pathogen *Colletotrichum lindemuthianum*. *Mol Microbiol* **64**, 68-82.

Jorgensen, K. (1998). A survey of pork, poultry, coffee, beer and pulses for Ochratoxin A. *Food Addit Contam* **15**, 550-554.

Jorgensen, K. & Jacobsen, J. S. (2002). Occurrence of ochratoxin A in Danish wheat and rye, 1992-1999. *Food Addit Contam* **19**, 1184-1189.

Kao, C. M., Katz, L. & Khosla, C. (1994). Engineered biosynthesis of a complete macrolactone in a heterologous host. *Science* **265**, 509-512.

Kennedy, J., Auclair, K., Kendrew, S. G., Park, C., Vederas, J. C. & Hutchinson, C. R. (1999). Modulation of polyketide synthase activity by accessory proteins during lovastatin biosynthesis. *Science* **284**, 1368-1372.

Kim, Y. T., Lee, Y. R., Jin, J., Han, K. H., Kim, H., Kim, J. C., Lee, T., Yun, S. H. & Lee, Y. W. (2005). Two different polyketide synthase genes are required for synthesis of zearalenone in *Gibberella zea*. *Mol Microbiol* **58**, 1102-1113.

Kuiper-Goodman, T. & Scott, P. M. (1989). Review: risk assessment of the mycotoxin ochratoxin A. *Biomed Environ Sci* **2**, 179-248.

Kuwayama, H., Shinji, O., Takahiro, M., Mariko, K., Hideko, U. & Yoshimasa, T. (2002). PCR-mediated generation of a gene disruption construct without the use of DNA ligase and plasmid vectors. *Nucleic Acids Res* **30**, 2e2.

Lee, T., Yun, S. H., Hodge, K. T., Humber, R. A., Krasnoff, S. B., Turgeon, G. B., Yoder, O. C. & Gibson, D. M. (2001). Polyketide synthase genes in insect- and nematode-associated fungi. *Appl Microbiol Biotechnol* **56**, 181-187.

Lui, D., Caloe, C., Biard, R. & Pedersen, J. (2000). Rapid mini preparation of fungal DNA for PCR. *J Clin Microboil* **38**, 471.

Machida, M., Asai, K., Sano, M. & Tanaka, T. (2005). Genome sequencing and analysis of Aspergillus oryzae. *Nature* **438**, 1157-1161.

Magan, N. & Olsen, M. (2004). *Mycotoxins in Food: Detection and Control*: Cambridge: DC/Woodhead Publishing Limited.

Metz, J. G., Roessler, P., Facciotti, D. & other authors (2001). Production of polyunsaturated fatty acids by polyketide synthase in both prokaryotes and eukaryotes. *Science* **293**, 290-293.

Nicholson, T. P., Rudd, B. A. M., Dawson, M., Lazarus, C. M., Simpson, T. J. & Cox, R. J. (2001). Design and utility of oligonucleotide gene probes for fungal polyketide synthases. *Chem Biol* **8**, 157-178.

O'Callaghan, J., Caddick, M. X. & Dobson, A. D. W. (2003). A polyketide synthase gene required for ochratoxin A biosynthesis in *Aspergillus ochraceus*. *Microbiol* **149**, 3485-3491.

Pel, H., J, W., Johannes, H. & other authors (2007). Genome sequencing and analysis of the versatile cell factory Aspergillus niger CBS 513.88. *Nature Biotechnol Pel* **25**, 221-231.

Petzinger, E. & Ziegler, K. (2000). Ochratoxin A from a toxicological perspective. *J Vet Pharmacol Ther* **23**, 91-98.

Sambrook, J., Fritsch, E. F. & Maniatis , T. (1989). Molecular cloning: a laboratory Manual. Cold Spring Harbor: Cold Spring Harbor laboratory press.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**, 503-517.

Tang, C. M., Cohen, J. & Holden, D. W. (1992). An *Aspergillus fumigatus* alkaline protease mutant constructed by gene disruption is deficient in extracellular elastase activity. *Molecular Microbiol* **6**, 1663-1671.

Torres, M., Balcells, M., Sala, N., Sanchis, V. & Canela, R. (1998). Bactericidal and fungicidal activity of *Aspergillus ochraceus* metabolites and some derivatives. *Pesticide Science* **53**, 9-14.

Varga, J., Rigo, K., Kocsube, S., Farkas, B. & Pal, K. (2003). Diversity of polyketide synthase gene sequences in *Aspergillus* species. *Res Microbiol* **154**, 593-600.

Yu, F., Zhu, X. & Du, L. (2005). Developing a genetic system for functional manipulations of FUM1, a polyketide synthase gene for the biosynthesis of fumonisins in *Fusarium verticillioides*. *FEMS Microbiol Lett* **248**, 257-264.

Zimmerli, B. & Dick, R. (1996). Ochratoxin A in table wine and grape-juice: Occurrence and risk assessment. *Food Addit Contam* **13**, 655-668.

Table 1: List of the oligonucleotide primers

Primers used in gene walking approach	
Primer Name	**Sequence (5'-3')**
LC3	GCNGARCAR ATGGAYCCNCA
LC5c	GTNGANGTNGCRTGNGCYTC
AT	YTG5GC5CCYTG5CC5GTDAA
ATR	CATRTGRTGIGARTGRTAIGC
ATR1	VDTYSAYDSSMRYMKHKS
LCF1	CTGATTCGTAGAATTTAC
LCF2	CTTCGGAATATCCGCCCA
LCF3	CTTCAGGTCAAGGGCGAG
Primers used in fusion PCR[††] , post transformation analysis and RT-PCR	
6- 5'lc12F	GCGATGCTCTTCGGGATG
3- 3'lc12R	TTC AAA CGA GCT AAC AGT
4- 5'*hph*R	<GTGCTCCTTCAATATCATCTTCTGT><ACTGCGAAGTGGAATGCGACTTGTG>
5- 3'*hph*F	<ATGCAGGAGTCGCATAAGGGAGAGC><GGCATTTGATGAGGCTGGTTTGGAC>
1- *hph*1F	GAATTCGAGCTCGGTACCCGGGGAT
2- *hph*1R	GCCAGCTTGCATGCCTGCAGGTCGA
LC12fullF	CAGATGGACCCGCAGCAG
LC12fullR	CATATTGACAACTGGCTG
SotLCF	TCTCCGCACCGCCATCAA
*hph*2F	CGGGGGCAATGAGATATGAAAAAG
*hph*2R	GAACCCGCTCGTCTGGCTAAG
TubF	CTCGAGCGTATGAACGTCTAC
TubR	AAACCCTGGAGGCAGTCGC
Aolc12F	AGAACGTGTATCATGCGCTCG
Aolc12R	GCATCGCGAAGTGGCTTGA

[††] The numbered primers are used in fusion PCR and two primers i.e. 4 and 5, are designed according to the procedure described by Kuwayama *et al*, 2002.

Figure legends and Figures

Fig. 1.

Alignment of the deduced amino acid sequence of *A. westerdijkiae aolc35-12* gene with other hybrid pks genes of; *A. niger* (GeneBank ID: An15g07920), *Penicillium marneffei* (GeneBank ID: ATCC 18224*A*), *A. terreus* (GeneBank ID:ATEG_03446), *A. clavatus* (GeneBank ID: XP_001268490.1) and *A. fumigatus* (GeneBank ID: EDP53793.1). Flash arrows indicate positions of different specific and degenerated primers (table 1) used in gene walking experiment

Fig. 2.

Transformation vector construction and disruption of *aolc35-12* gene: (a) Modified form of PCR- mediated generation of gene disruption technique (Kuwayama *et al.*, 2002) was used to construct TopLC12*hph* vector. Arrows marked by numbers indicate different primers (table 1). The blue colour tilted lines behind primers 4 and 5 indicate *hph* tails (b) Protoplasts of *A. westerdijkiae* were prepared and *aolc35-12* gene was disrupted using TopoLC12*hph* vector to obtain *aoΔlc35-12* mutants. Different colours on the *aoΔlc35-12* indicate different functional domains i.e. *Escherichia coli* hygromycin B phosphotransferase gene, β-ketoacyl synthase (KS), acyltransferase (AT). The small red line below *aoΔlc35-12* indicates position of radioactively labelled probe lc12p: *aolc35-12* gene specific probe amplified by using primer pair SotLCF / LC12fullR. Position of the restriction sites of *HindIII* is indicated by triangle.

Fig. 3.

Screening of the *aolc35-12* disrupted transformants: (a) Genomic DNA of wild type *A. westerdijkiae, ao*Δ*lc35-12a, ao*Δ*lc35-12b, ectlc12* and ao+lc35-12 transformants were digested with *HindIII* restriction enzyme and probed with radioactively labelled DNA fragments of *aolc35-12* gene "lc12p", amplified through primer pair SotLCF / LC12fullR. In the wild-type a 3.7 kb band was detected, while in the transformants *ao*Δ*lc35-12a* and *ao*Δ*lc35-12b* the 3.7 kb band is replaced by the expected 5 kb band. In transformant *eclc12* the 3.7 kb band is intacted and an additional band was revealed

showing an ectopic insertion of the replacement construct. In the genetically complemented mutant *ao+lc35-12* the 5 kb band is replaced by the wild-type 3.7 kb band. (b) Characteristic fluorescence of OTA under UV light observed in wild type *A. westerdijkiae* CYA culture, and the absence of fluorescence in *ao*Δ*lc35-12* transformants.

Fig. 4.

HPLC traces and UV spectra of mellein (eluted at 27 min) and OTA (eluted at 34 min) in wild type *A. westerdijkiae* NRRL 3174 (black) and *ao*Δ*lc35-12* (blue) and *ao+lc35-12* mutants (pink).

Fig. 5.

Differential expression of OTA-PKS genes in *A. westerdijkiae*: Profile of *aoks1* gene expression (first panel) and *aolc35-12* gene expression (second panel) in wild-type *A. westerdijkiae, aolc35-12* gene expression in *ao*Δ*ks1* (third panel) and *aoks1* gene expression in *ao*Δ*lc35-12* (fourth panel) by RT-PCR. Beta tubuline was used as loading control (bottom panel).

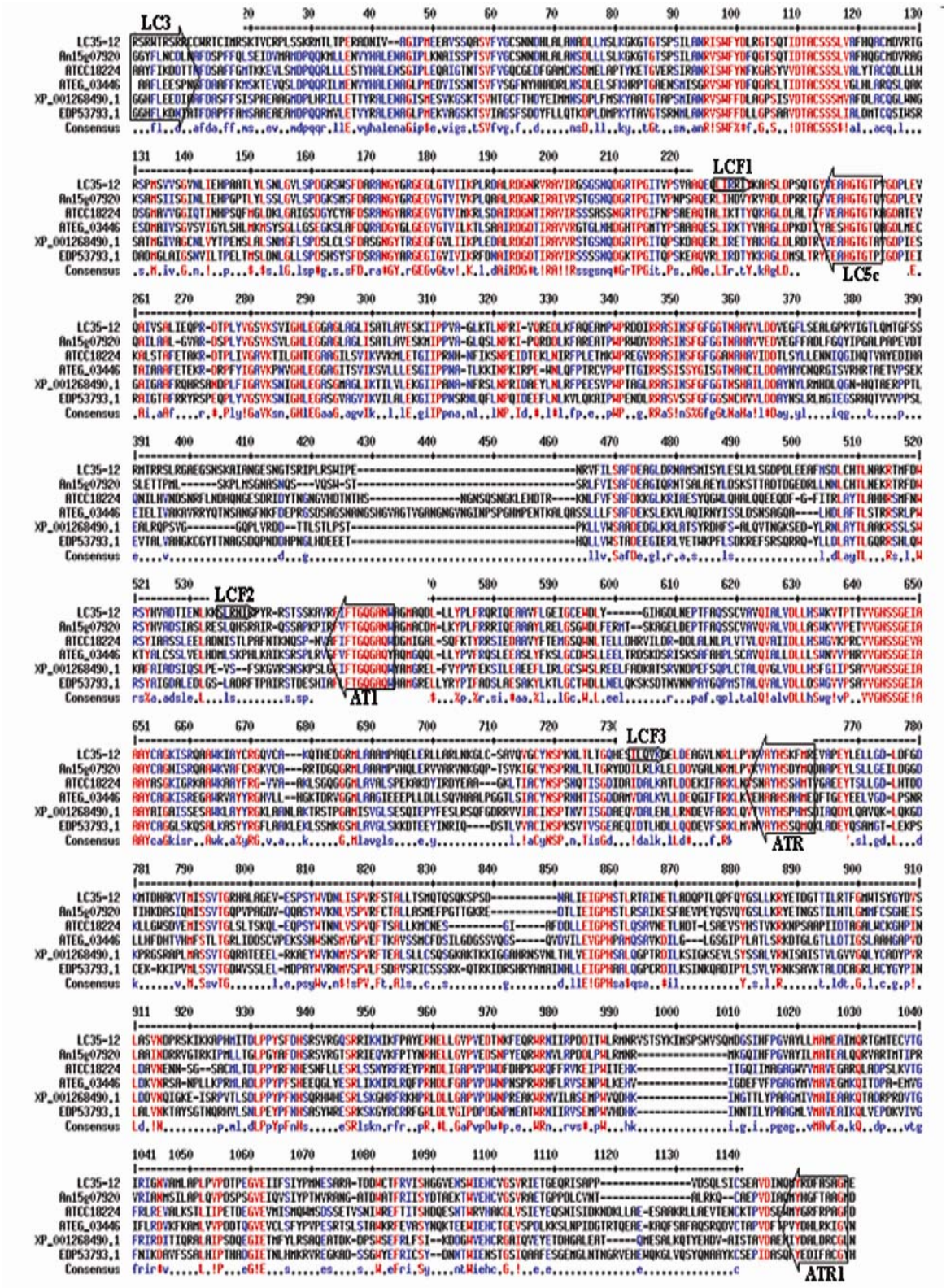
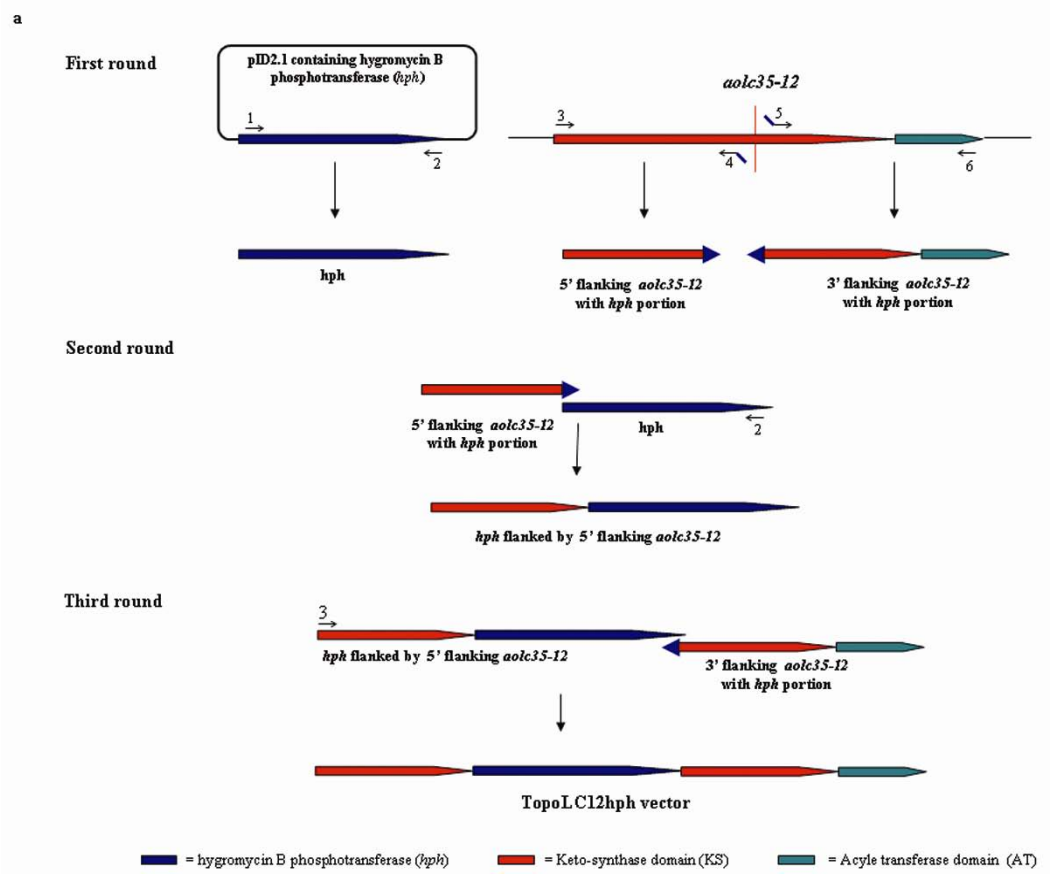


Figure 1.


a
First round
pID2.1 containing hygromycin B phosphotransferase (hph)
aolc35-12
hph
5' flanking aolc35-12 with hph portion
3' flanking aolc35-12 with hph portion
Second round
5' flanking aolc35-12 with hph portion
hph
hph flanked by 5' flanking aolc35-12
Third round
hph flanked by 5' flanking aolc35-12
3' flanking aolc35-12 with hph portion
TopoLC12hph vector
= hygromycin B phosphotransferase (hph)
= Keto-synthase domain (KS)
= Acyle transferase domain (AT)

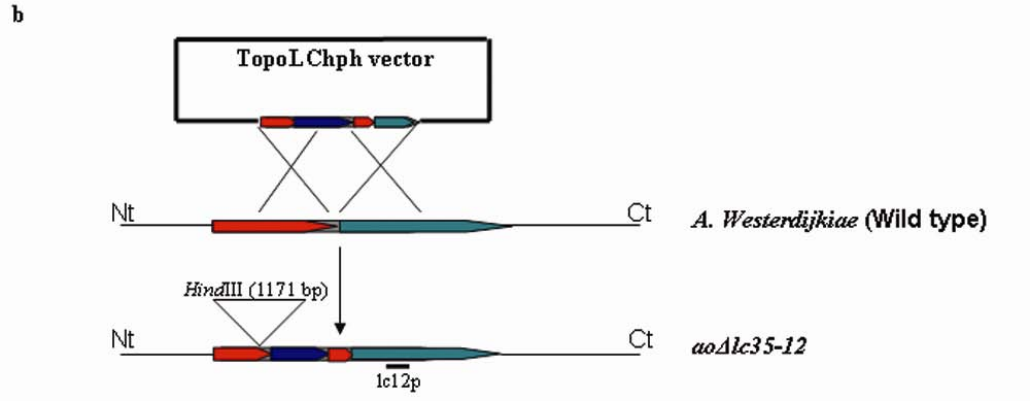

b
TopoLChph vector
Nt
Ct
A. Westerdijkiae (Wild type)
HindIII (1171 bp)
Nt
Ct
aoΔlc35-12
lc12p

Figure 2.

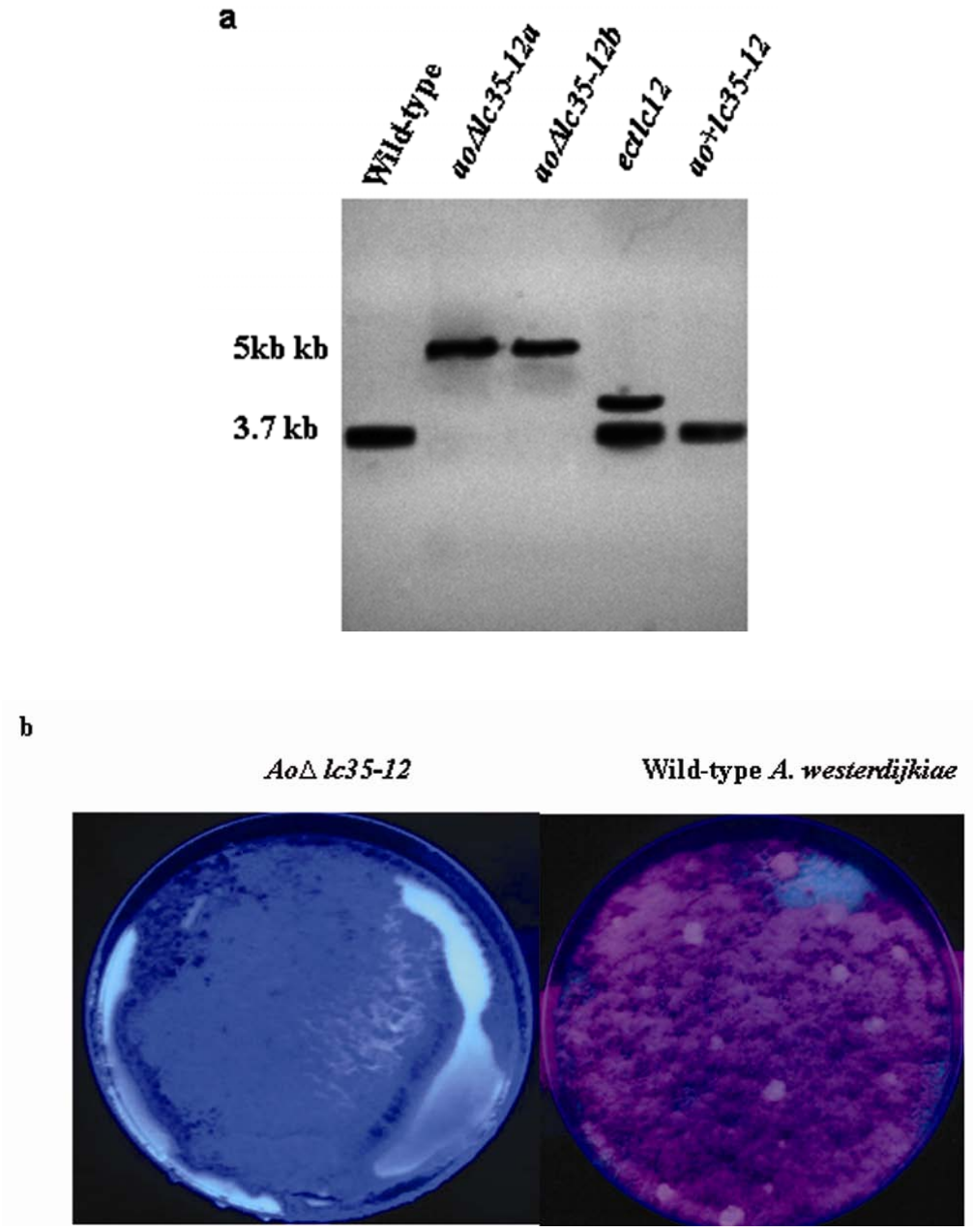

a
Wild-type
ao∆lc35-12a
ao∆lc35-12b
ectlc12
ao+lc35-12
5kb kb
3.7 kb
b
Ao∆ lc35-12
Wild-type A. westerdijkiae

Figure 3.

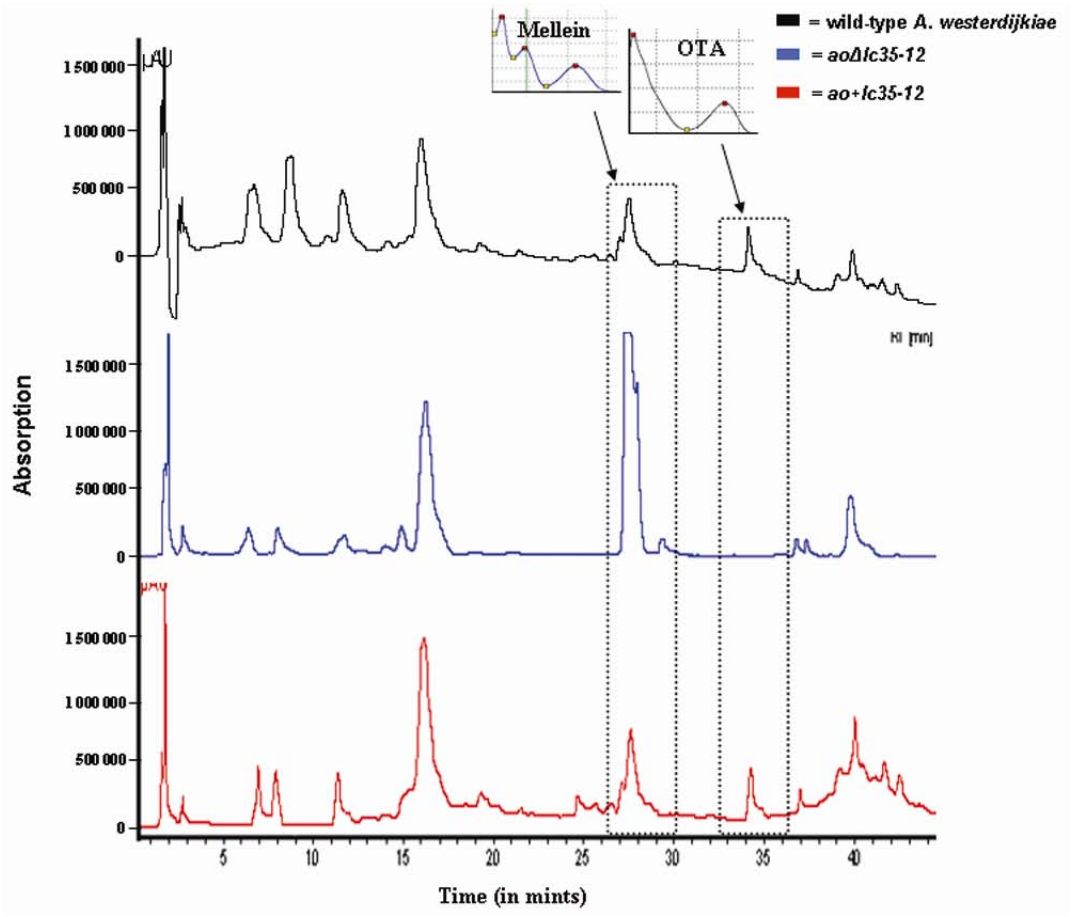


Figure 4.

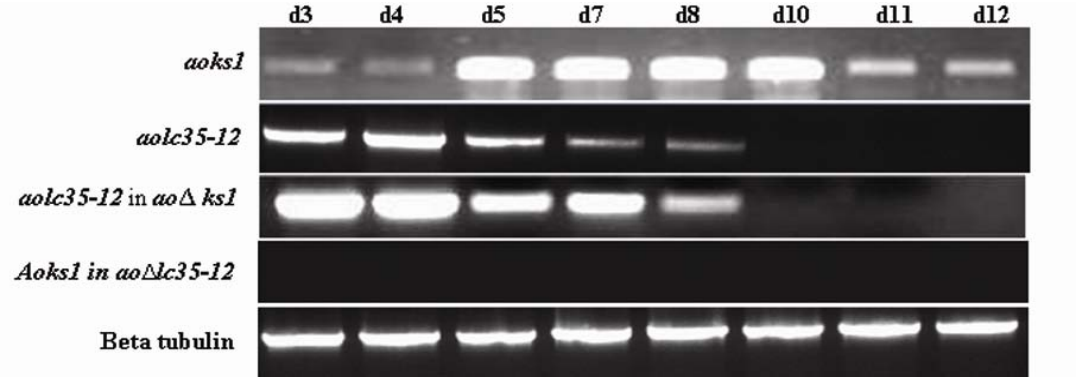


Figure 5.

Chapter III

Article 3 : Published in Fungle Genetics and Biology (2009), Volume 46, 742-749.

3.3 Cloning and characterization of novel methylsalicylic acid synthase gene involved in the biosynthesis of isoasperlactone and asperlactone in *Aspergillus westerdijkiae*

Nafees Bacha[1], Huy Phong Dao[1], Ali Atoui[2], Florence Mathieu[1], John O'Callaghan[3], Olivier Puel[4], Thierry Liboz[1], Alan D.W. Dobson[3], and Ahmed Lebrihi[1‡‡]

[1]Université de Toulouse, Institut National Polytechnique de Toulouse, Laboratoire de Génie Chimique UMR5503 (CNRS/INPT/UPS), Ecole Nationale Supérieure Agronomique de Toulouse, 1, avenue de l'Agrobiopôle, BP 32607, 31326 Castanet Tolosan, France.

[2] Lebanese Atomic Energy Commission-CNRS, P.O. Box 11-8281, Riad El Solh, 1107 2260 Beirut, Lebanon.

[3]Department of Microbiology and Environmental Research Institute, University College Cork, National University of Ireland Cork, Cork, Ireland.

[4]Laboratoire de Pharmacologie-Toxicologie, Institut National de Recherche Agronomique, BP 3, 180 chemin de Tournefeuille, 31931 Toulouse cedex 9, France.

Running title: Asperlactone/Isoasperlactone PKS from *A. westerdijkiae*

‡‡ Corresponding author

Tel: 00 33 (0)5 62 19 39 44

Fax: 00 33 (0)5 62 19 39 01

E-mail: lebrihi@ensat.fr

Abstract

Aspergillus westerdijkiae is the main producer of several biologically active polyketide metabolites including isoasperlactone and asperlactone. A 5298 bp polyketide synthase gene "*aomsas*" has been cloned in *Aspergillus westerdijkiae* by using gene walking approach and RACE-PCR. The predicted amino acid sequence of *aomsas* shows an identity of 40 to 56 % with different methylsalicylic acid synthase genes found in *Byssochlamys nivea, P. patulum, A. terreus* and *Streptomyces viridochromogenes*. Based on the reverse transcription PCR and kinetic secondary metabolites production studies, *aomsas* expression was found to be associated with the biosynthesis of isoasperlactone and asperlactone. Moreover an *aomsas* knockout mutant "*ao*Δ*msas*" of *A. westerdijkiae*, not only lost the capacity to produce isoasperlactone and asperlactone, but also 6-methylsalicylic acid. The genetically complemented mutant *ao+msas* restored the biosynthesis of all the missing metabolites. Chemical complementation through the addition of 6-methylsalicylic acid, aspyrone and diepoxide to growing culture of *ao*Δ*msas* mutant revealed that these compounds play intermediate roles in the biosynthesis of asperlactone and isoasperlactone.

Key words: *Aspergillus westerdijkiae*, polyketide synthase, asperlactone, isoasperlactone, 6-methylsalicylic acid.

1. Introduction

Aspergillus westerdijkiae is a producer of several biologically active polyketide metabolites including isoasperlactone and asperlactone (Atoui *et al.*, 2006; Balcells *et al.*, 1995; Gaucher & Shepherd, 1968; Torres *et al.*, 1998). These two metabolites belong to the partially reduced or methylsalicylic acid (MSA) type polyketide group and have same chemical structure but are different stereochemically. Isoasperlactone and asperlactone are on one hand efficient antimicrobial agents (Rosenbrook & Carney, 1970; Torres *et al.*, 1998), and on the other hand they contain ovicidal activities against *Nezara viridula* (Balcells et al., 1995; Balcells et al., 1998). It has been reported that asperlactone and isoasperlactone presented strong anti-bacterial and anti-fungal activities when compared with other antimicrobial agents (Balcells *et al.*, 1998).

It has been previously demonstrated that during the biosynthesis of MSA type polyketides, the first step is the formation of 6-methylsalicylic acid (6-MSA) by the condensation of one molecule of acetyl-coA and three molecules of malonyl-coA (David et al., 1995). This reaction is catalyzed by a multifunctional enzyme system known as methylsalicylic acid synthase (MSAS). This enzyme has been reported to catalyze several MSA type polyketide compounds like patuline in *Penicillium patulum* (Beck et al., 1990) and *Byssochlamys fulva* (Puel et al., 2007) and avilamycin in *Streptomyces viridochromogenes* (Gaisser et al., 1997).

Not much is known about the biosynthetic pathways of isoasperlactone and asperlactone, except for a hypothetical scheme proposed by James & Andrew (1991). According to this scheme, the isomeric metabolites aspyrone, isoasperlactone and asperlactone are derived from a common biosynthetic precursor, the diepoxide. James & Andrew (1991) further stated that asperlactone is formed directly from the diepoxide, while isoasperlactone is formed from diepoxide via aspyrone pathway (Fig 1).

Many PKS genes have been sequenced in filamentous fungi including *A. fumigatus* and *A. niger* (Metz et al., 2001; Niermanm et al., 2005; Pel et al., 2007; Sebastian et al., 2007). It is therefore possible to use the most conserved region among these PKS genes (Fig. 2) as primers for a PCR based cloning strategy. For this purpose, mainly the ketosynthase (KS) and acyle transferase (AT) domains i.e. the most conserved domains

among different PKSs (Fig. 2), have been utilized. The previously designed pairs of degenerated primers KS1/ KS2, LC1 / LC2c and LC3/LC5c (Bingle et al., 1999; Nicholson et al., 2001) has been already utilized in our laboratory to clone eight KS domains from different PKSs (Atoui et al., 2006), including one from MSAS in *A. westerdijkiae*. Recently one of these PKS genes has been found to be involved in the biosynthesis of ochratoxine A (Bacha *et al.*, 2009a).

In this paper, we report sequencing and functional characterisation of the first MSAS type PKS gene "*aomsas*" identified in *A. westerdijkiae*. It is involved in the biosynthesis of two pharmacologically important lactonic metabolites i.e. isoasperlactone and asperlactone. We also demonstrate that 6- methylsalicylic acid (6-MSA), aspyrone and diepoxide are intermediates in their biosynthetic pathway.

2. Materials and Methods

2.1. Fungal strain and culture conditions

A. westerdijkiae NRRL 3174 strain was grown for sporulation at 25°C on potato dextrose agar for 7 days. Spores were collected using a solution of 0.01% (v/v) Tween 80, counted by using Thoma Bright line counting chamber (Optick labor) , 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 synthetic medium (SAM) at 25 °C for 2 to 18 days, without shaking. The composition of SAM (per liter of distilled water) was: 3 g NH_4NO_3, 26 g K_2HPO_4, 1 g KCl, 1 g $MgSO_4.7H_2O$, 10 mL mineral solution (composition per liter of distilled water: 70 mg $Na_2B_4O_7.10H_2O$, 50 mg $(NH_4)_6$ $Mo_7O_{24}.4H_2O$, 1000 mg $FeSO_4.7H_2O$, 30 mg $CuSO_4.5H_2O$, 11 mg $MnSO_4.H_2O$, 1760 mg $ZnSO_4.7H_2O$), and 50 g glucose. The pH of the medium was adjusted to 6.5 by the addition of 2 N HCl. Mycelium was harvested by filtration through a 0.45µM filter, grounded in liquid nitrogen and then stored at -80 °C before nucleic acid extraction. Secondary metabolites were extracted from filtrates of 2 to 18 days old cultures medium. Three replications of each sample were analyzed.

2.2. Nucleic acid extraction

Rapid method of genomic DNA extraction (Lui et al., 2000) was used for transformants screening by PCR. Large quantity genomic DNA was extracted by CTAB extraction method (Gardes & Bruns, 1993). The quality and quantity of DNA were estimated by measuring OD 260 nm / OD 280 nm and OD 260 nm respectively.

Total RNA was extracted from *A. westerdijkiae* using the Tri-reagent (Euromedex France) DNA / RNA/ Protein extraction kit. The quality and quantity of RNA was checked by the OD 260 nm / OD 280 nm ratio and agarose gel electrophoresis according to standard protocols (Sambrook et al., 1989).

2.3. Cloning of aomsas gene in A.westerdijkiae

Degenarated primers based Gene walking approach and RACE-PCR was used to clone *aomsas* gene in *A. westerdijkiae*. Figure 2 mark positions while table 1 list all the primers used during this study. The degenerated primer pair LC3 / LC5c (Bingle et al., 1999)

designed for ketosynthase (KS) domains of MSAS type PKSs was used to isolate putative PKS gene fragment of 700 bp (Atoui et al., 2006). The degenerated primers MS2 and MS4 targeting the conserved regions GVSAMGFPW and GVVHAAGV were further used with specific primer MS1 allowed by PCR to clone an additional 2 kb of the gene. Using the same strategy, an additional 1.3 kb downstream sequence of the *aomsas* gene was subsequently cloned. Finally the 5′ and 3′ extremity of the *aomsas* gene were subsequently amplified by using a RACE-PCR approach on cDNA (kit supplied by Invitrogen with 5′-primer and 3′-primer). Each time the PCR products obtained were cloned in pCR2.1-Topo vector (Invitrogen) and sequenced. Alignments of the sequenced fragments were performed to search for consensus.

2.4. PCR reaction and sequencing

PCR was performed with the *Taq* recombinant polymerase (Invitrogen, USA). Amplification was carried out in a 50 µl reaction mixture containing: 5 µl of *Taq* polymerase 10× buffer, 1·5 µl of 50 mM $MgCl_2$, 1 µl of dNTP 10 mM of each (Promega), 1 µM of each primer, 1·5 U of *Taq*, about 200 ng of DNA genomic, H_2O up to 50 µl. Reaction conditions were: 94 °C for 4 min, (94 °C for 45 s, 53 °C for 45 s and 72 °C for 1 min) × 30 cycles followed by an incubation at 72 °C for 10 min. The amplified products were examined by 1 % (w/v) agarose gel. The PCR products were cloned into pCR2.1-Topo vector according to the supplier's instructions. Sequencing of the fragments was performed by Genomexpress (Grenoble, France).

2.5. 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://prodes.toulouse.inra.fr/multalin/multalin.html.

2.6. Reverse transcription-PCR reaction

The expression of aomsas gene in A. westerdijkiae was examined by using reverse treascription-PCR (RT-PCR) with two specific primers AoLC35-2L and AoLC35-2R designed from the KS domain of aomsas gene (Table 1). For RT-PCR, total RNA was

treated with DNase I (Promega) to remove DNA contamination. cDNA was synthesized from each sample with Advantage RT- for-PCR Kit (BD Biosciences) according to the supplier's manual. cDNA amplification were performed using Taq recombinant polymerase (Invitrogen,USA). Beta tubulin was used as positive control using primers TubF and TubR (Table 1).

2.7. Plasmid construction and transformation

To construct TopoMS*hph*, the *aomsas* gene replacement vector, a 1995 bp amplified fragment containing *Sal1* restriction site was obtained using MSdF and MSdR primers (Fig. 4a; table 1). This fragment was ligated into pCR2.1-Topo plasmid generating the TopoMS plasmid. TopoMS and pID2.1 plasmids were restricted using *Sal*I enzyme. The *hph* cassette (2.4 kb) obtained from pID2.1 was ligated with the restricted TopoMS plasmid to produce TopoMS*hph* vector, in which the 5` and 3`-ends of *hph* cassette are flanked by *aomsas* gene fragments. To construct the complementation vector pAN-MSAS, PCR primers MSdF1 and MSdR1 were designed to amplify a 5 kb aomsas gene fragment. The amplified fragment was cloned in pAN8-1 plasmid containing phleomycine resistance cassette.

The aomsas gene inactivation and complementary mutant production were achieved by preparing protoplasts of the corresponding strains and transforming with TopoMS*hph* (Fig. 3b) and pAN-MSAS respectively as previously described (Jaoanne et al., 2007; O'Callaghan et al., 2003). 40 mg/mL lysing enzyme (Sigma) was used for the preparation of protoplasts.

2.8. Screening of the transformants

The *aomsas* disrupted mutants (*ao⊿msas*) were initially selected on YES medium (20 g/l of yeast extract, 1 M sucrose) supplemented with 150 µg/mL of hygromycin B, while the genetically complemented mutants (*ao+msas*) were selected on YES medium supplemented with 120 µg/mL phleomycin. These mutants were further screened through PCRs, using two *hph* gene specific primers *hph*2R and *hph*2F (Table 1) and *aomsas* gene specific primers MSdF1, MSdR1 and MSdR. Total genomic DNA from wild type *A. westerdijkiae, ao*Δ*msas* and *ao+msas* strains were digested with enzymes *Bal*1 and *Sma*1 and were subjected to southern hybridization (Southern, 1975) to confirm *aomsas* gene

disruption and genetic complementation. These restriction enzyme were chosen because they cut the 5´- flanking region of the *aomsas* (position 2054 bp and 2850 bp) but do not cut the *hph* cassette or 3´- flanking end of the *aomsas* gene (Fig. 4b). The digested DNA was then transferred to nylon membrane (Amersham, France) and probed with radioactively labelled DNA fragments of *aomsas1* gene "PM" amplified through primer pair 3´MsF/3´MsR (probe position is shown in Fig. 4b).

2.9. Extraction of secondary metabolites

For secondary metabolites extraction of wild type *A. westerdijkiae*, *ao*Δ*msas* and *ao+msas* mutants, 30 mL filtrate sample of the culture medium was acidified with 200 µL of 12 N HCl, mixed with 30 mL chloroform and vigorously shaken for 10 min. The solvent phase was then decanted, dried under vacuum and re-dissolved in 0.5 mL methanol. 20 µL of the sample was then further analysed by HPLC.

2.10. High-performance liquid-chromatography (HPLC) analysis

The HPLC apparatus consisted of a solvent delivery system, with both fluorescence (λ_{ex} = 332 nm; λ_{em} = 466 nm) and UV detectors (BIO-TEK, Milan, Italy). The analytical column used was a 150 x 4.6 mm Uptisphere 5 µm C18 ODB fitted with a guard column of 10 x 4 mm. The column temperature was 30 °C. Kroma 3000 (BIO-TEK) was the data acquisition system. Injections were done with an auto-injector (BIO-TEK, Milan, Italy) and the injection volume was 20 µL. The samples were analyzed by linear gradient elution using 0.2 % glacial acetic acid in 99.8 % water (v/v) (A) and 100 % acetonitrile (HPLC grade) (B). The crude extract was analyzed using a linear elution gradient over 45 min at a flow rate of 1 ml/min, starting from 10 to 50% solvent B over the first 30 min, continued by a linear gradient to 90 % of B in 5 min, followed by an isocratic flow of 90% solvent B for 8 min, and a return to initial conditions over the last 2 min of the run.

Secondary metabolites of *A. westerdijkiae* were detected by comparing the elution time and maximum absorption of UV with the standard isoasperlactone and asperlactone (Alexis-biochemicals France) and 6- methylsalicylic acid (ACROS, USA). These standards were used at a concentration of 10µg/mL. 6- methylsalicylic acid (maximum absorption λ_{max} = 205, 240, 302 nm) were released at 5 min and isoasperlactone/

asperlactone (maximum absorption λ_{max} = 220 nm) were released between 7 min and 9 min.

*2.11. Chemical complementation study of ao*Δ*msas mutants*

The *ao*Δ*msas* mutants were inoculated in 250 mL Erlenmeyer flask containing 100 mL SAM medium and incubated at 25°C. After 72 hours 0.7 mM each of 6-MSA, aspyrone and diepoxide were added independently to separate growing cultures of *ao*Δ*msas* mutants and again incubated at 25°C without shaking. After 4 days from precursor addition secondary metabolites were extracted for HPLC analyses.

2.12. Sequence accession number

The sequence obtained was deposited in Genbank under the accession number AY540947.

3. Results

3.1. aomsas is a MSAS type polyketide gene of A. westerdijkiae

With the intent of extending the 700 bp KS domain fragment of a MSAS type PKS gene identified by Atoui et al, (2006) in *A. westerdijkiae*, we adopted the degenerated primers based gene walking and RACE-PCR techniques. The specific primers MS1, MS3, MS5 and MS6 and degenerated primers MS2 and MS4 (table1) allowed the sequencing of a complete 5298 bp *aomsas* gene (Fig. 2). The *aomsas* gene displayed a unique open reading frame (ORF) of 1766 amino acids. Alignment of the amino acid sequence of *aomsas* with other PKS gene displayed an identity of 56 % to 6-MSAS gene involved in the biosynthesis 6-MSA in *Byssochlamys nivea*, 54 % to 6-MSAS gene involved in the biosynthesis of patulin in *P. patulum* and 50 % to a hypothetical 6-MSAS gene in *A. terreus* (Fig. 2). The *aomsas* also displayed a significant identity of 40 % with the PKS responsible for orsellinic acid biosynthesis in *Streptomyces viridochromogenes*. Like other 6-MSAS gene, *aomsas* contained characteristic conserved domains of fungal type I PKSs (Fig. 2). These domains were (from N terminus to C terminus) β-ketoacyl synthase, acyltransferase, dehydratase, β-ketoacyl reductase, and an acyl carrier protein. No thioesterase or enoyl-reductase domains were found in *aomsas* gene.

3.2. Asperlactone and isoasperlactone productions and aomsas gene expression in A. westerdijkiae

In liquid synthetic medium (SAM), without shaking at 25° C, *A. westerdijkiae* presented two exponential growth phases from day 3 to day 5 (early exponential growth phase) and day 6 to day 9 (late exponential growth phase), followed by a stationary phase from day 9 onward (Fig. 3a). Asperlactone and isoasperlactone were simultaneously excreted from day 4 and reach a maximum level at day 9 and day 10 respectively. We have further observed that 6-MSA was transitorily produced in very low quantity between day 6 and day 8 and then disappeared (Fig. 3a).

Tracking the transcriptional signals of *aomsas* gene during different growth stages of *A. westerdijkiae* in SAM medium, we observed that expression of the gene started from day 3 onwards, stayed constant at maximum level between day 4 and 9 and then stopped after day 11 (Fig. 3b).

3.3. aomsas gene code for 6-MSA, isoasperlactone and asperlactone in A. westerdijkiae

Following transformation of *A. westerdijkiae* with TopoMS*hph* vector (Fig. 4b), one hundred and twenty transformants were obtained. Forty transformants were screened by two consecutive PCRs on genomic DNA in order to monitor the integration of *hph* gene into the genome of *A. westerdijkiae*. In only two out of the selected forty transformants the *hph* cassette was detected by the two PCR tests (5% transformation efficiency was obtained). Digested genomic DNA from the wild type *A. westerdijkiae* and the two *ao*Δ*msas* transformants were subsequently analyzed by southern blotting. In the wild type *A. westerdijkiae* a signal corresponding to 2.8 kb fragment of *aomsas* gene was observed when its digested genomic DNA was probed with PM (Fig. 4c, lane 1). Probing of the aomsas disrupted mutants i.e. *ao*Δ*msas1* and *ao*Δ*msas2*, with PM probe resulted into an expected signal of 5.2 kb (2.8 kb correspond to *aomsas* +2.4 kb correspond to *hph* cassette) (Fig. 4c, lane 3 and 4 respectively). We have also observed one ectopic mutant i.e. *ectMS*, where the transformation construct was inserted into a non targeted region (Fig. 4c, lane 2).

Wild type *A. westerdijkiae*, *ao*Δ*msas1*, *ao*Δ*msas2* and *ectMS*, were grown on solid CYA and liquid SM medium, no difference was observed in their colony sizes, growth patterns, visual pigments and conidial counts. Filtrate of the 10 days old culture of wild type *A. westerdijkiae*, *ao*Δ*msas* and *ectMS* mutants were used to extract secondary metabolites. The HPLC profiles of the extracted secondary metabolites revealed that inactivation of *aomsas* gene by the insertion of *hph* cassette disrupt the production of isoasperlactone, asperlactone (Fig. 5a) and the transitory biosynthesis of 6-MSA. No impact of the ectopic insertion was observed on the biosynthesis of secondary metabolites (data not shown).

To confirm the role of *aomsas* gene, genetic complementation of the two *ao*Δ*msas* mutants were performed with pAN-MSAS transformation vector (containing *aomsas* gene fragment and phleomycine gene cassette). Filtrate of the 10 days old culture of positively tested complemented mutant *ao+msas* were used to extract secondary metabolites. HPLC profile of *ao+msas* mutant revealed that re-insertion of *aomsas* gene restored the productions of asperlactone, isoasperlactone and 6-MSA (Fig. 5a).

3.4. 6-MSA, diepoxide and aspyrone are intermediates of isoasperlactone and asperlactone

Chemical complementation was performed by independent incorporation of 6-MSA (0.7 mM), diepoxide (0.7mM) and aspyrone (0.7mM) to separate growing cultures of *ao*Δ*msas* mutants. HPLC profile and UV spectra of the chemically complemented mutants revealed that addition of 6-MSA, diepoxide and aspyrone produced similar effects and restored the biosynthesis of both isoasperlactone and asperlactone (Fig. 5b).

4. Discussion

From the analyses of fungal PKS gene sequences, Bingle et al. (1999) suggested that these genes could be divided into two subclasses designated as WA-type and MSAS-type or partially reduced type. They designed two pairs of degenerated primers i.e. LC1 / LC2c and LC3 / LC5c for the amplification of fungal PKS genes in each of these subclasses. We have previously utilized LC3 / LC5c pair of primer to clone a 700 bp KS domain of *aomsas* gene in *A. westerdijkiae* (Atoui et al., 2006). After prolongation of this fragment by gene walking approach to a complete 5298 bp gene and alignment in database with other PKS genes, we have observed that the amino acid sequence of *aomsas* has high degree similarities to fungal MSAS type PKS genes (Fig. 2). The different conserved domains identified in *aomsas* further confirmed that this gene belongs to the MSAS-type subclass (Fig. 2). These observations are in accordance to the findings of Bingle et al. (1999), who stated that the degenerated pair of primers LC3 / LC5c has the capability to amplify fungal MSAS-type PKS genes.

To understand the role of *aomsas* gene in *A. westerdijkiae*, we first investigated the kinetic production of secondary metabolites and the expression of the *aomsas* gene. We have observed that both transcriptional signals of *aomsas* and biosynthesis of asperlactone, isoasperlactone and 6-MSA occur within the exponential growth phase of *A. westerdijkiae* (Fig. 3). These observations suggest that *aomsas* could be related to the biosynthesis of asperlactone and / or isoasperlactone and / or 6-MSA. It has been reported in the literature that MSAS-type PKS genes are involved in the biosynthesis of several olyketide metabolites varying greatly in their functions. An MSAS-type PKS gene *6msas* has been found to be involved in the biosynthesis myctoxine patuline in *P. patulum, P. urticae* and *Byssochlamys fulva* (Beck et al., 1990; Bu'Lock et al., 1968; Puel et al., 2007). Another MSAS gene *AviM* has been found to be involved in the biosynthesis of antibiotic avilamycin in *S. viridochromogenes* (Gaisser et al., 1997). The MSAS gene has also been reported to induce the production plant defense compound salicylic acid in tobacco (Nasser et al., 2001). Although *A. westerdijkiae* is a non-producer of patuline, avilamycin and salicylic acid but this do not preclude the possibility of having an MSAS type PKS gene in its genome. A precedent of such a situation is provided by Geisen,

1996, who used a multiplex PCR method to identify sequences homologous to aflatoxin biosynthesis gene in the non-producing species of *Aspergillus* and *Penicillium* (Geisen, 1996).

Insertional inactivation remained the key technique for functional characterization of various fungal pks genes (Atoui *et al.*, 2006; Bacha *et al.*, 2009a; O'Callaghan *et al.*, 2003). So we followed similar technique to produce *aomsas* knockout mutants "*ao*Δ*msas1*" and "*ao*Δ*msas2*". These mutants were found deficient in the biosynthesis of two end product lactonic metabolites i.e. isoasperlactone and asperlactone (Fig. 4a), and one compound of intermediate nature i.e. 6-MSA. These observations confirmed that *aomsas* played an important role in the biosynthesis of 6-MSA, isoasperlactone and asperlactone. We further proved these results by performing genetic complementation, where the genetically complemented mutant *ao+msas* restored the biosynthesis of all the missing metabolites (Fig. 4a).

It has been previously demonstrated through NMR studies that diepoxide and aspyrone are intermediates in the biosynthetic pathway of isoasperlactone and asperlactone in *A. melleus* (Fig. 1) (James & Andrew, 1991). These authors further stated that asperlactone is directly biosynthesized from the diepoxide, while in the biosynthesis isoasperlactone the diepoxide is first converted to aspyrone and then aspyrone to isoasperlactone. They also suggested an alternate rout for the biosynthesis isoasperlactone without the involvement of aspyrone. Chemical complementation of the asperlactone / isoasperlactone deficient mutant i.e. *ao*Δ*msas*, through incorporation of 6-MSA, diepoxide and aspyrone clearly shown that, these products are intermediates in the biosynthetic pathway of asperlactone and isoasperlactone (Fig.5b). Indeed each of the incorporated metabolite restored simultaneously the biosynthesis of both asperlactone and isoasperlactone. Aspyrone and diepoxide are never observed in the culture medium of wild type *A. westerdijkiae*. This could probably be due to a quick conversion of these compounds to their final products (asperlactone and isoasperlactone), avoiding an intracellular accumulation and extracellular excretion.

Based on our results and the findings of James and Andrew, (1991) we proposed a biosynthetic scheme for the biosynthesis of isoasperlactone and asperlactone (Fig. 1). According to this scheme, during the early biosynthetic steps *aomsas* gene induces the

production of 6-MSA, which is then converted to diepoxide. The diepoxide could then follow two routes, one that leads directly to the production of asperlactone while the second leads to the production of isoasperlactone via aspyrone pathway. We have observed during chemical complementation experiment that even incorporation of aspyrone without diepoxide restored the production asperlactone and isoasperlactone in *ao*Δ*msas* mutant. Previously James and Andrew, (1991) did not proposed this scheme; they proposed aspyrone as intermediate of isoasperlactone but not of asperlactone. Hence we proposed that diepoxide and aspyrone could be enter-convertible compounds which could lead to the production of both asperlactone and isoasperlactone. Our observations are in accordance to the previous findings, suggesting that lactonic metabolites asperlactone and isoasperlactone could be derived from a common biosynthetic precursor, a diepoxy intermediate (Sorensen & Simpson, 1986).

This new *aomsas* gene can be used for further experiments, as a starting material for the identification of asperlactone / isoasperlactone gene cluster.

ACKNOWLEDGMENTS

The authors are grateful to the Higher Education Commission of Pakistan for according PhD scholarship to Mr. Nafees Bacha.

References

Atoui, A., Dao., P., Mathieu, F., Lebrihi, A., 2006. Amplification and diversity analysis of Ketosynthase domains of putative polyketide synthase gene in *Aspergillus ochraceus* and *Aspergillus carbonarius* producers of ochratoxin A. Mol Nutr Food Res. 50, 448-493.

Bacha, N., Atoui, A., Mathieu, F., Liboz, T., Lebrihi, A., 2009. Aspergillus westerdijkiae polyketide synthase gene "aoks1" is involved in the biosynthesis of ochratoxin A. Fungal Genetics and Biology. 46, 77-84.

Balcells, M., Canela, R., Coll, j., Sanchis, V., Torres, M., 1995. Effect of fungal metabolites and some derivatives against *Tribolium castaneum* (Herbst) and *Nezara viridula* (L.). Pestic sci. 45, 319-323.

Balcells, M., Canela, R., Sala, N., Sanchis, V., Torres, M., 1998. Bactericidal and fungicidal activity of *Aspergillus westerdijkiae* metabolites and some derivatives. Pestic sci. 53, 00009-00014.

Beck, J., Ripka, S., Siegner, A., Schiltz, E., Schweizer, E., 1990. The multifunctional 6-methylsalicylic acid synthase gene of *Penicillium patulum*. Its gene structure relative to that of other polyketide synthases. Eur J Biochem. 192**,** 487–498.

Bingle, L. E., Simpson, T. J., Lazarus, C. M., 1999. Ketosynthase domain probes identify two subclasses of fungal polyketide synthase genes. Fungal Genet Biol. 26, 209-223.

Bu'Lock, J. D., Sheperd, D., Winstanley, D. J., 1968. Regulation of 6-methylsalicylate and patulin synthesis in Penicillium urticae. Can J Microbiol. 15**,** 279 - 285.

David, J. B., Eckhart, S., David, A. H., Chaitan, K., 1995. Expression of a functional fungal polyketide synthase in the bacterium *Streptomyces coelicolor* A3(2). J Bacteriol. 177, 4544–4548.

Gaisser, S., Trefzer, A., Stockert, S., Kirschning, A., Bechthold, A., 1997. Cloning of an avilamycin biosynthetic gene cluster from *Streptomyces viridochromogenes* Tü57. J of Bacteriol. 179**,** 6271-6278.

Gardes, M., Bruns, T. D., 1993. TS primers with enhanced specificity for Basidiomycetes-applicatin to the identification of mycorrhizae and rusts. Mol Ecol. 2, 113-118.

Gaucher, G. M., Shepherd, M. G., 1968. Isolation of orsellinic acid synthase. Biochem and Biophys Res Com. 32, 664-671.

Geisen, R., 1996. Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. System Appl Microbiol. 19, 388-392.

James, S., Andrew, C. S., 1991. 17O NMR in biosynthetic studies: aspyrone, asperlactone and isoasperlactone, metabolites of *Aspergillus melleus.* J Chem Soc., Chem Commun. 1106-1108.

Jaoanne, W. S. H., Corentin, H., Nafees, B., Richard, O. C., Claude, L., Giséle, B., Michel, R., Pierre, R., Bernard, D., 2007. Regulation and role of the STE12-like transcription factor from the plant pathogen *Colletotrichum lindemuthianum*. Mol Microbiol. 64, 68-82.

Lui, D., Caloe, C., Biard, R., Pedersen, J., 2000. Rapid mini preparation of fungal DNA for PCR. J Clin Microboil. 38, 471.

Metz, J. G., Roessler, P., Facciotti, D., other authors, 2001. Production of polyunsaturated fatty acids by polyketide synthase in both prokaryotes and eukaryotes. Science. 293, 290-293.

Nasser, Y., Daniel, J., Altier, E., Barbour, A., Cigan, L., Christopher, J. S., 2001. Production of 6-Methylsalicylic Acid by Expression of a Fungal Polyketide Synthase Activates Disease Resistance in Tobacco. plant cell. 13**,** 1401-1410.

Nicholson, T. P., Rudd, B. A. M., Dawson, M., Lazarus, C. M., Simpson, T. J., Cox, R. J., 2001. Design and utility of oligonucleotide gene probes for fungal polyketide synthases. Chem Biol. 8, 157-178.

Niermanm, W. C., et al., 20058. Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. Nature. 438, 1151-1156.

O'Callaghan, J., Caddick, M. X., Dobson, A. D. W., 2003. A polyketide synthase gene required for ochratoxin A biosynthesis in *Aspergillus ochraceus*. Microbiol. 149, 3485-3491.

Pel, H., et al., 2007. Genome sequencing and analysis of the versatile cell factory Aspergillus niger CBS 513.88. Nature. Biotechnol. Pel. 25**,** 221-231.

Puel, O., S.Tadrist, Delafoege, M., Oswald, I. P., Lebrihi, A., 2007. The inability of *Byssochlamys fulva* to produce patulin is related to absence of 6-methylsalicylic acid synthase and isoepoxydon dehydrogenase genes. Int J Food Microbiol. 115, 131-139.

Rosenbrook, W. J., Carney, R. E., 1970. New metabolite from an unidentified *Aspergillus species*. Tetrahedron Letters. 22, 1867-70.

Sambrook, J., Fritsch, E. F., Maniatis , T. 1989. Molecular cloning: a laboratory Manual. Cold Spring Harbor laboratory press, Cold Spring Harbor.

Sebastian, B., Julia, S., Kirstin, S., Corinna, L., Axel, A. B., Christian, H., 2007. Genomics-driven discovery of PKS-NRPS hybrid metabolites from *Aspergillus nidulans*. Nature chem biol. 3, 213-217.

Sorensen, W. G., Simpson, J., 1986. Toxicity of penicillic acid for rat alveolar macrophages in vitro. Environ Res. 41, 505-513.

Southern, E. M., 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol. 98, 503-517.

Torres, M., Balcells, M., Sala, N., Sanchis, V., Canela, R., 1998. Bactericidal and fungicidal activity of *Aspergillus ochraceus* metabolites and some derivatives. Pesticide Science. 53, 9-14.

Table 1. Oligonucleotide primers

Primer name	Sequence (5′-3′)
LC3	GCIGA(A/G)CA(A/G)ATGGA(T/C)CCICA
LC5c	GTIGAIGTIGC(G/A)TGIGC(T/C)TC
MS1	GCCGCCGGCGTTGACCCGATGAC
MS2	CGGCCIAAGGIAA(T/C)ICCITG
MS3	CTTAGCAAATCATTCACCATGGAC
MS4	(C/T)T(G/C)(A/G)(T/C)(C/T)(A/T)TCCAGCACGCCIGC
MS5	ATCCAAACGCTAGACCAACTCGGC
MS6	CATTTTCGATCGCCTGCCATGCC
MSdF1	ATGCCTTTCTTGGATCCGTCCTCG
MSdR1	CTAGGCAGCTAGTTTCTCCGCGAAC
MSdF	CCGATCGTGTTCAGATCTTGACCTTCCTGATG
MSdR	ACTAAGTTGGGGATGTCCTGAGATGACGAGA
AoLC35-2R	CTGGAGGATCTCGCTGATGT
AoLC35-2L	TTTGATCGACCATTGTGTGC
3’MsR :	CGGGGGCAATGAGATATGAAAAAG
3’MsF :	GAACCCGCTCGTCTGGCTAAG
*hph*2R	CTGGATGCAGCAACCTCTAT
*hph*2F	CTGAGATGACGAACTTGAC
TubF	CTCGAGCGTATGAACGTCTAC
TubR	AAACCCTGGAGGCAGTCGC

Figure legends and figures

Fig. 1.

Proposed biosynthetic scheme of isoasperlactone and asperlactone in *A. westerdijkiae*: Based on our results and the study of James & Andrew, (1991), we proposed a hypothetical biosynthetic scheme of isoasperlactone and asperlactone biosynthesis. Straight arrow lines indicate our proposed scheme, dotted arrow lines indicate biosynthetic scheme of James & Andrew, (1991), while dashed arrow line indicate steps that are shared in our proposed biosynthetic scheme and in the scheme of James & Andrew, (1991).

Fig. 2

Alignment of the deduced amino acid sequence of *aomsas* gene with other MSAS type PKS gene: 6-MSAS involved in the biosynthesis 6-MSA in *Byssochlamys nivea*(Accession N°: AAK48943), 6-MSAS involved in the biosynthesis of patulin in *p. patulum* (Accession N°: CAA39295), 6-MSAS in *A. terreus* (Accession N°: AAC49814) and PKS responsible for orsellinic acid biosynthesis in *Streptomyces viridochromogenes* (Accession N°:AAK83194). Red boxes indicate conserved regions of different functional domains. Flash arrows indicate positions of different specific and degenerated primers (table 1) used in gene walking experiment.

Fig. 3.

Kinetic production of secondary metabolites and expression of *aomsas* gene in *A. westerdijkiae*. (a) Kinetic production of isoasperlactone, asperlactone and 6-MSA in a growing culture of *A. westerdijkiae* at 25°C in synthetic medium during a time course of 2-18 days. (b) Profile of *aomsas* gene expression (upper panel) by RT-PCR. Beta tubuline was used as loading control (bottom panel).

Fig. 4.

Schematic representation of *aomsas* gene disruption in *A. westerdijkiae* : (a) Using primer pair MsdF/MsdR (table 1), 1995 bp *aomsas* fragment containing *Sal*1 restriction site (indicated by triangle) was amplified from a 5.298 kb *aomsas* gene. PCR product was

cloned into pCR2.1-Topo plasmid to generate plasmid TopoMS. pID2.1 plasmid vector was restricted with *Sal*1 (indicated by triangle) to obtain *hph* cassette (2.4 kb). TopoMS was restricted with *Sal*1 and ligated with *hph* cassette to genarate TopoMS*hph* transformation vector. Different colours on the *aomsas* gene indicate different functional domains i.e. β-ketoacyl synthase {KS), acyltransferase (AT), dehydratase (DH), β-ketoacyl reductase (KR), and an acyl carrier protein (ACP). (b) Protoplasts of *A. westerdijkiae* were prepared and *aomsas* gene was disrupted using TopoMS*hph* vector to obtain *ao⊿msas* mutants. The small red line below *ao⊿msas* indicates position of radioactively labelled probe i.e. PM: *aomsas* gene specific probe amplified by using primer pair AoLC35-2R /AoLC35-2L. Positions of the restriction sites of *Sma*1 and *Sal*1 are indicated by triangles. (c) Genomic DNA of wild type *A. westerdijkiae, ao*Δ*msas* and *ao+msas* transformants were digested with *Sma*1 and *Bal*1 restriction enzymes and probed with radioactively labelled *aomsas* fragment (PM). In the wild-type a 2.8 kb band was detected. In transformant *ectMS* the 2.8 kb band is intacted and an additional band was revealed showing an ectopic insertion of the replacement construct. In the transformants *ao*Δ*msas1* and *ao*Δ*msas1* the 2.8 kb band is replaced by the expected 5.2 kb band. In the genetically complemented mutant *ao+msas* the 5.2 kb band is replaced by the wild-type 2.8 kb band.

Fig. 5.

Secondary metabolites study: (a) Secondary metabolites were extracted from 10 days old cultures of wild-type, *ao*Δ*msas* and *ao+msas* strains of *A. westerdijkiae*. HPLC traces of the lactonic metabolites i.e isoasperlactone (eluted at 6 min) and asperlactone (eluted at 9 min) were compared (b) Separate growing cultures of *ao*Δ*msas* mutant in synthetic medium at 25 °C were independently complemented with 0.7 mM 6- each of methylsalicylic acid, aspyrone and diepoxide. Secondary metabolites were extracted from 8 days old cultures and analysed by tracking the HPLC traces.

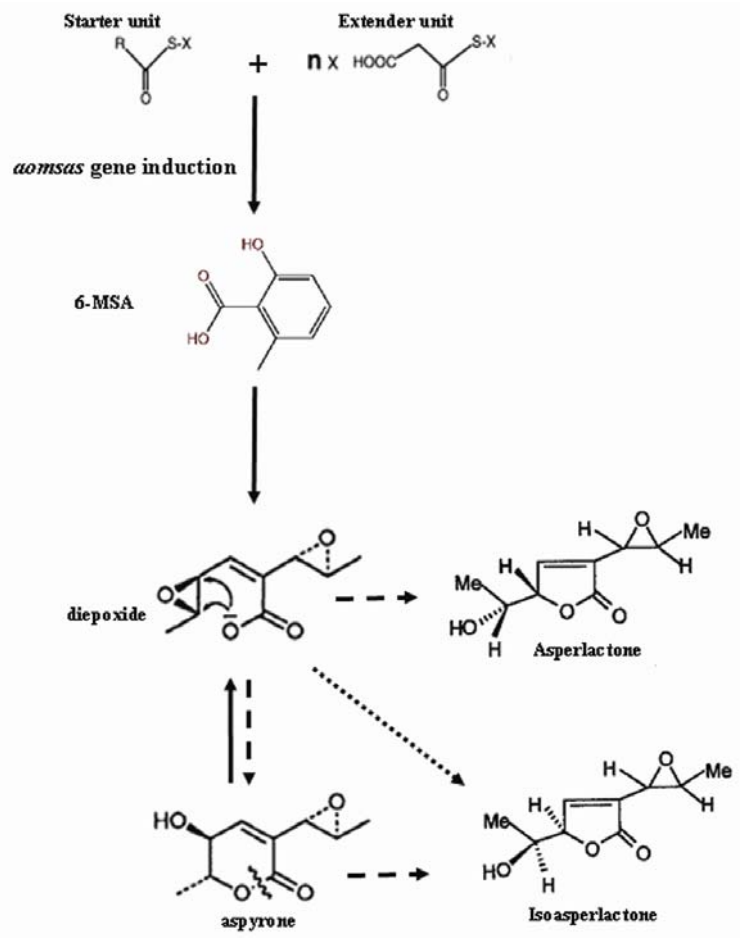

Starter unit
Extender unit
aomsas gene induction
6-MSA
diepoxide
Asperlactone
aspyrone
Isoasperlactone

Figure 1

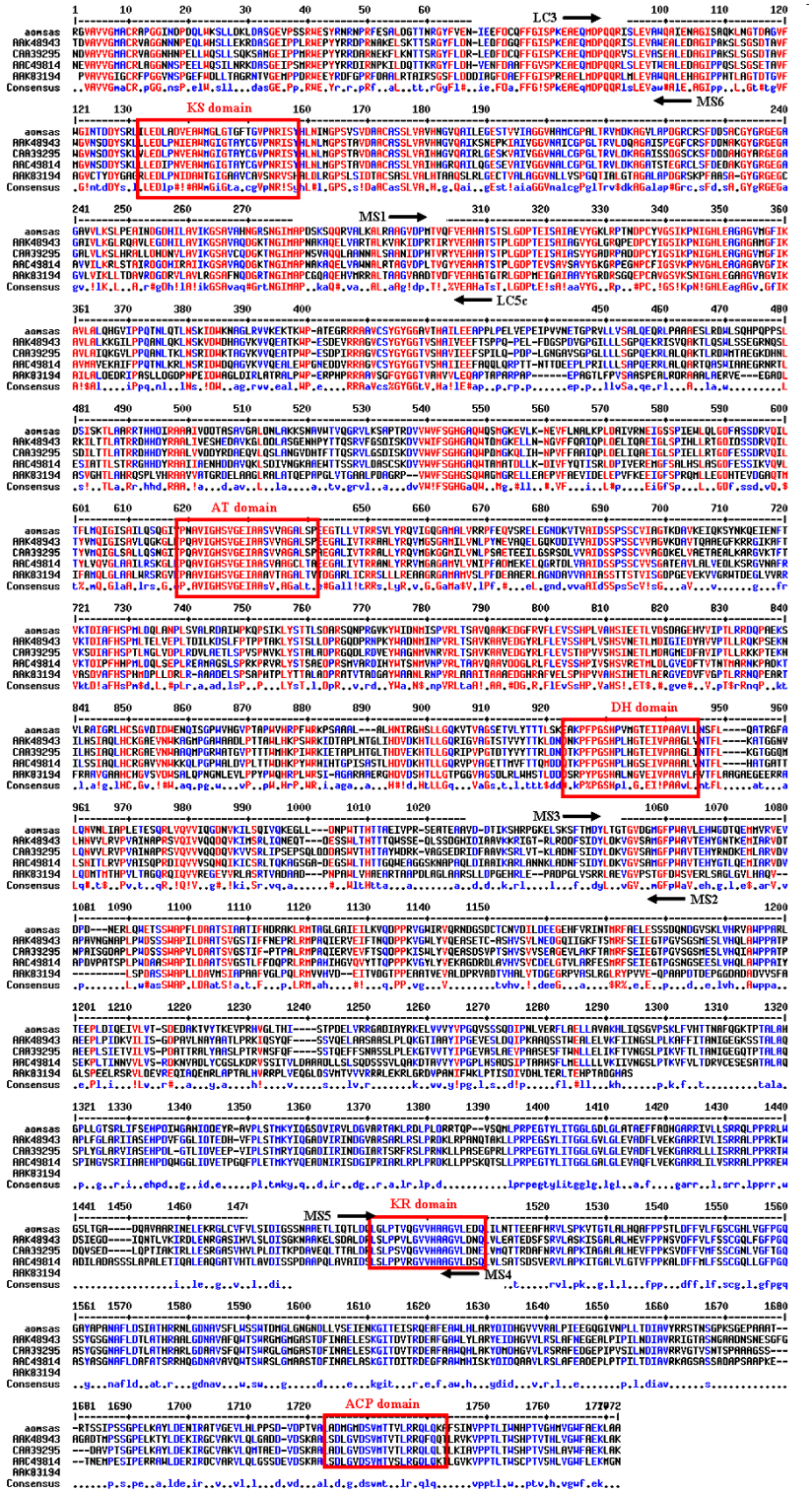

LC3
MS6
KS domain
MS1
LC5c
AT domain
DH domain
MS3
MS2
MS5
KR domain
MS4
ACP domain

Figure 2

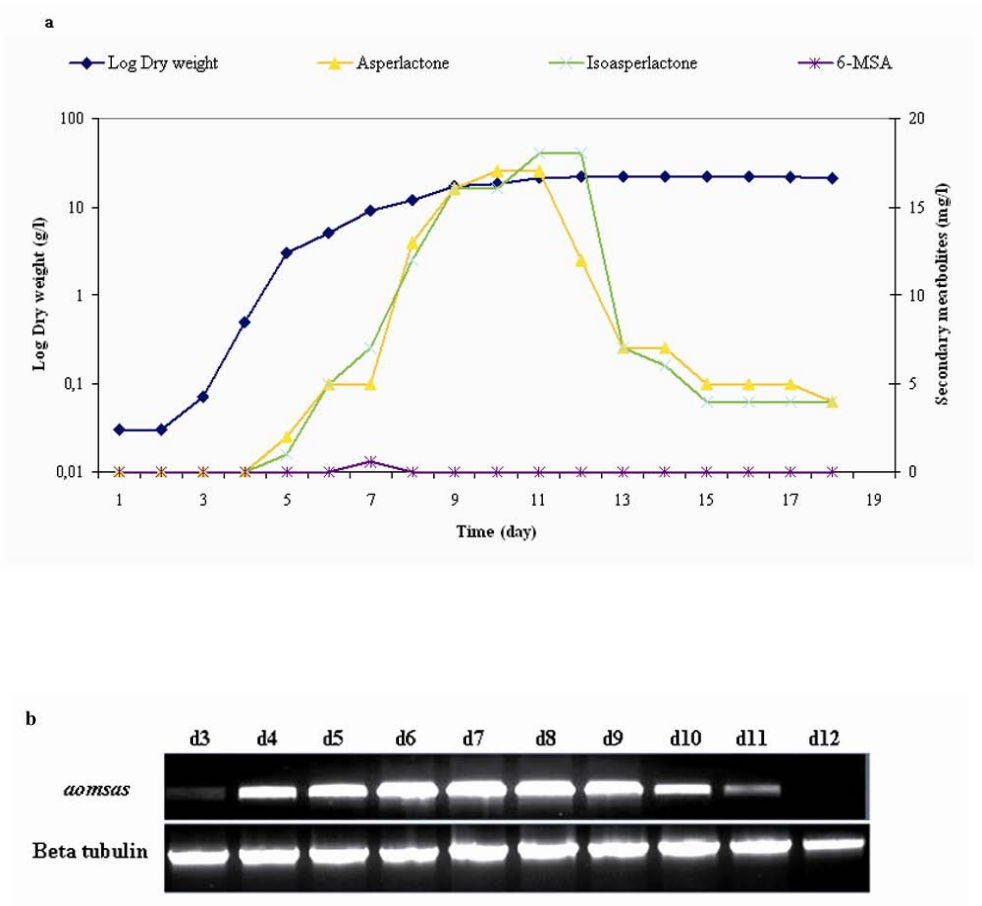

a
Log Dry weight
Asperlactone
Isoasperlactone
6-MSA
Log Dry weight (g/l)
Secondary meatbolites (mg/l)
Time (day)
b
d3
d4
d5
d6
d7
d8
d9
d10
d11
d12
aomsas
Beta tubulin

Figure 3

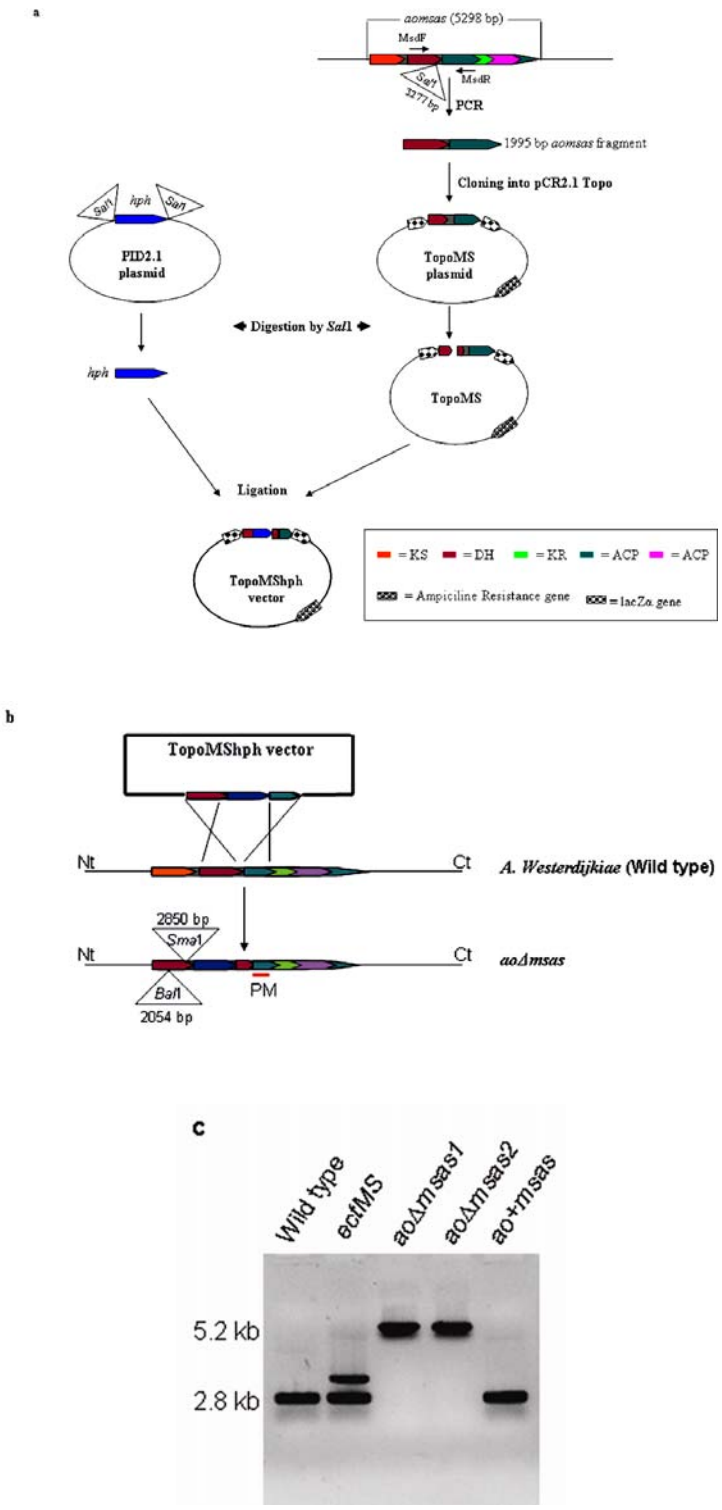

a
aomsas (5298 bp)
MsdF
MsdR
Sal1
3271 bp
PCR
1995 bp aomsas fragment
Cloning into pCR2.1 Topo
Sal1
hph
Sal1
PID2.1 plasmid
TopoMS plasmid
Digestion by Sal1
hph
TopoMS
Ligation
TopoMShph vector
= KS
= DH
= KR
= ACP
= ACP
= Ampiciline Resistance gene
= lacZα gene
b
TopoMShph vector
Nt
Ct
A. Westerdijkiae (Wild type)
2850 bp
Sma1
Nt
Ct
aoΔmsas
Ball
2054 bp
PM
c
Wild type
ectMS
aoΔmsas1
aoΔmsas2
ao+msas
5.2 kb
2.8 kb

Figure 4

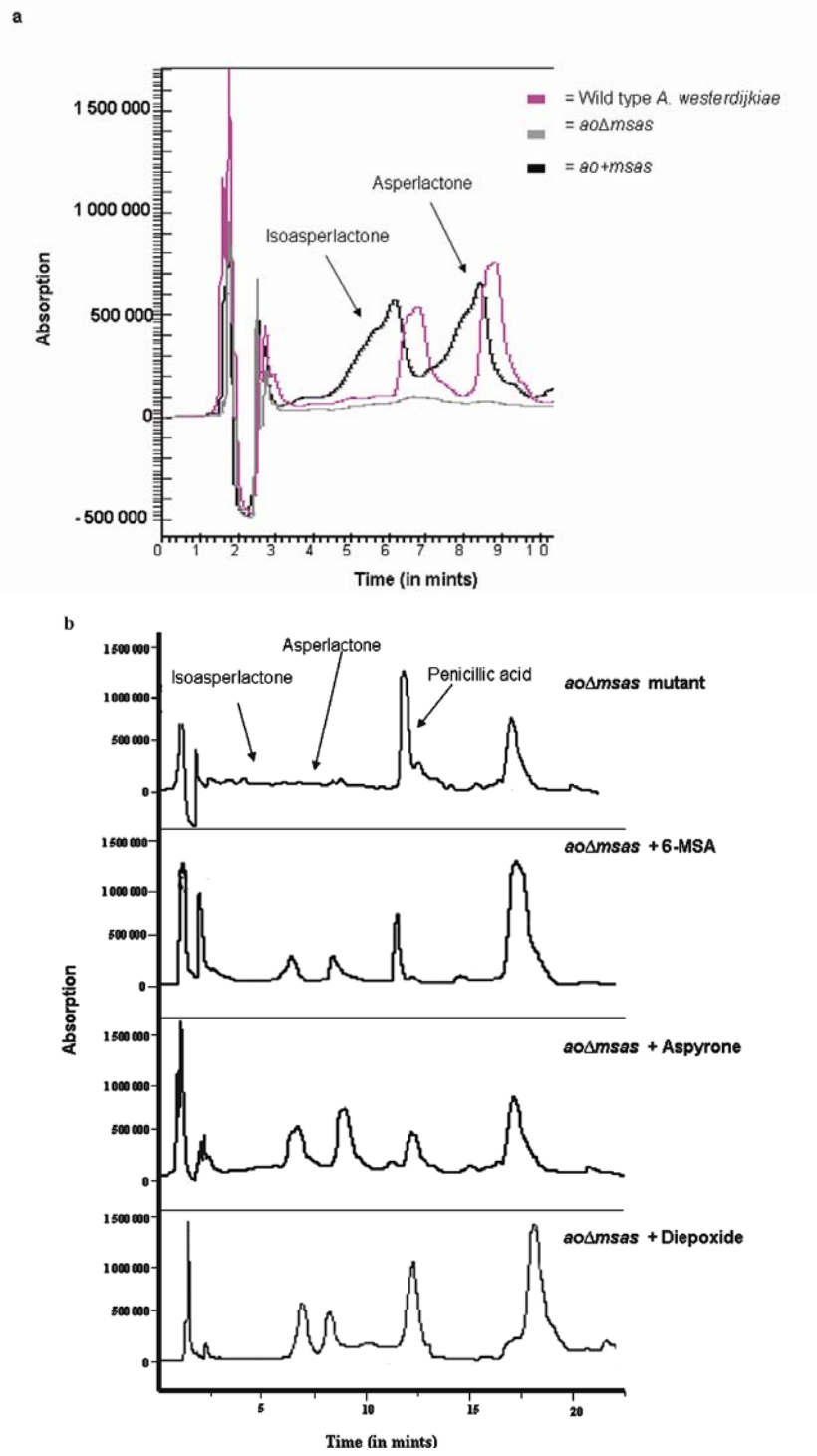

a
= Wild type A. westerdijkiae
= aoΔmsas
= ao+msas
Asperlactone
Isoasperlactone
Absorption
1 500 000
1 000 000
500 000
0
- 500 000
0 1 2 3 4 5 6 7 8 9 10
Time (in mints)
b
Asperlactone
Isoasperlactone
Penicillic acid
aoΔmsas mutant
aoΔmsas +6-MSA
aoΔmsas + Aspyrone
aoΔmsas + Diepoxide
Absorption
5 10 15 20
Time (in mints)

Figure 5

Chapter IV

Article 4 : Underconsideration in Microbial Biotechnology

3.4 A polyketide synthase – non ribosomal peptide synthase gene deferentially controls penicillic acid, isoasperlactone and asperlactone genes in *Aspergillus westerdijkiae*

Nafees Bacha[1], Florence Mathieu[1], Thierry Liboz[1] and Ahmed Lebrihi[18]

[1]Université de Toulouse, Institut National Polytechnique de Toulouse, Laboratoire de Génie Chimique UMR5503 (CNRS/INPT/UPS), Ecole Nationale Supérieure Agronomique de Toulouse, 1, avenue de l'Agrobiopôle, BP 32607, 31326 Castanet Tolosan, France.

Running head: lactone metabolites gene in *A. westerdijkiae*

[8] Corresponding author

Tel: 00 33 (0)5 62 19 39 44

Fax: 00 33 (0)5 62 19 39 01

E-mail: lebrihi@ensat.fr

Abstract

Penicillic acid (PA), isoasperlactone and asperlactone are biologically active secondary metabolites produced by several strains of *Penicillium* and *Aspergillus*. By using genome walking approach, we partially cloned the *aolc35-6* gene (1999 bp) in *Aspergillus westerdijkiae*, which is identified as a polyketide synthase – non ribosomal peptide synthase (PKS-NRPS). A mutant of *A. westerdijkiae* "*ao⊿lc35-6*", in which *aolc35-6* gene was disrupted by hygromycin B phosphotransferase gene, lost the capacity to produce PA, isoasperlactone and asperlactone. The genetically complemented mutant *ao+lc35-6* restored the biosynthesis of all the missing metabolites. Further chemical complementation studies by the addition of either orsellinic acid or 6-methylsalicylic acid to the growing cultures of the mutant *ao⊿lc35-6* restored the production of PA and isoasperlactone / asperlactone respectively. This indicated that orsellinic acid and 6-methylsalicylic acid are intermediates in the biosynthetic pathways of PA and isoasperlactone / asperlactone respectively in *A. westerdijkiae*. Gene expression assay further confirmed that *aolc35-6* diferentially controls the expression of penicillic acid and asperlactone / isoasperlactone genes in *A. westerdijkiae*.

Key words: *Aspergillus westerdijkiae*, polyketide synthase / non-ribosomal peptide synthase, penicillic acid, asperlactone, isoasperlactone, 6-methylsalicylic acid, orsellinic acid.

Introduction

Aspergillus westerdijkiae which was recently dismembered from *A. ochraceus* (Frisvad *et al.*, 2004a), naturally contaminates a variety of food commodities e.g. cereals, coffee, vines and forages etc prior to harvest and more commonly during storage (Antonia *et al.*, 2008; Christensen & Tuthill, 1985; Kozakiewicz, 1989). This fungus produces several biologically active secondary metabolites like ochratoxin A, penicillic acid (PA), isoasperlactone and asperlactone etc (Atoui *et al.*, 2006; Balcells *et al.*, 1995; Torres *et al.*, 1998). These metabolites are polyketide compounds varying greatly in their functions (Mosbach, 1969), e.g. PA possesses antibacterial activities against both Gram-positive and Gram-negative bacteria and has a high herbicide activity against various field weeds (Madhyastha *et al.*, 1994; Michito *et al.*, 1996). This is also an effective antifungal agent especially against *Phytophthra spp* (Kang & Kim, 2004). While isoasperlactone and asperlactone are efficient antimicrobial agents (Rosenbrook & Carney, 1970; Torres *et al.*, 1998) and also contain ovicidal activities against *Nezara viridula* (Balcells *et al.*, 1995; Balcells *et al.*, 1998).

Not much is known about the biosynthetic pathways of PA, isoasperlactone and asperlactone except for certain hypothetical schemes (Axberg & Catenbeck, 1975; James & Andrew, 1991; Sekiguchi *et al.*, 1987). Axberg & Catenbeck 1975, explained biosynthesis of PA as; orsellinic acid leads to 2-O-methylorsellinic acid leads to 1,4-dihydroxy-6-methoxy-2-methylbenzene leads 6-methoxy-2-methyl-benzoquinone (1,4) leads to PA. This biosynthetic pattern was further modified by Sekiguchi *et al*. (1987) demonstrating that 6-methyl-1,2,4-benzenetriol is also an intermediate of PA. Based on the NMR study, James & Andrew (1991) demonstrated that the isomeric metabolites aspyrone, isoasperlactone and asperlactone are derived from a common biosynthetic precursor, the diepoxide. They further stated that asperlactone is formed directly from the diepoxide, while isoasperlactone is formed from diepoxide via espyrone pathway. More recently chemical complementation of aspyron to the asperlactone/isoasperlactone negative mutant culture revealed that aspyron is equally important for the biosynthesis of asperlactone (Bacha *et al.*, 2009b).

Recently, several genomes of filamentous fungi have been sequenced. Genome-sequencing projects for *A. fumigatus* and other filamentous fungi have revealed the

presence of approximately 30 to 50 gene clusters per species, which include genes that encode enzymes catalyzing the production of polyketide compounds i.e. non-ribosomal peptide synthase (NRPS) and polyketide synthase (PKS) (Metz *et al.*, 2001; Niermanm *et al.*, 2005; Sebastian *et al.*, 2007) (fungal genome resource list at http://www.fgsc.net). Many of the filamentous fungi sequenced to date also contain at least one gene that encodes a "hybrid" enzyme possessing domains typical of both PKS and NRPS. While such hybrid genes were described some time ago for bacteria (Du & Shen, 2001), only recently have the products of a few of the predicted fungal hybrid *PKS-NRPS* genes been determined. These include *fusA*, which is required for synthesis of a precursor of fusarin C in *Fusarium moniliforme* and *F. venenatum* (Song *et al.*, 2004) a gene required for equisetin biosynthesis in *F. heterosporum* (Sims *et al.*, 2005) and a gene that encode stenellin synthetase from *Beauveria bassiana* (Eley *et al.*, 2007).

The database of the already cloned *PKS* (Atoui *et al.*, 2006; Bingle *et al.*, 1999; Nicholson *et al.*, 2001) and *NRPS* (de Crecy-Lagard *et al.*, 1997) genes have been used to identify highly conserved regions among these genes. These regions were then used to design degenerated primers that could help in the identification and functional characterization of novel polyketide genes.

Here we present the first study of the identification and functional characterization of a hybrid PKS-NRPS gene "*aolc35-6*", involved in the biosynthesis of polyketide metabolites i.e. PA, isoasperlactone and asperlactone, in *A. westerdijkiae*. We also provide evidence about the intermediate role of 6-methylsalicylic acid (6-MSA) and orsellinic acid in the biosynthesis of these metabolites.

Materials and Methods

Fungal strain and culture conditions

A. westerdijkiae NRRL 3174 strain was grown for sporulation at 25°C on potato dextrose agar for 7 days. Spores were collected using a solution of 0.01% (v/v) Tween 80, counted by using Thoma Bright line counting chamber, and stored at -20°C in 25% (v/v) glycerol before use. For metabolites production conidia were inoculated (density ~ 10^6 /mL) into 250 mL Erlenmeyer flasks containing 100 mL synthetic medium (SAM) at 25 °C for 2 to 18 days, without shaking. The composition of SAM (per liter of distilled water) was: 3 g NH_4NO_3, 26 g K_2HPO_4, 1 g KCl, 1 g $MgSO_4.7H_2O$, 10 mL mineral solution (composition per litre of distilled water: 70 mg $Na_2B_4O_7.10H_2O$, 50 mg $(NH_4)_6 Mo_7O_{24}.4H_2O$, 1000 mg $FeSO_4.7H_2O$, 30 mg $CuSO_4.5H_2O$, 11 mg $MnSO_4.H_2O$, 1760 mg $ZnSO_4.7H_2O$), and 50 g glucose. The pH of the medium was adjusted to 6.5 by the addition of 2 N HCl. Mycelium was harvested by filtration through a 0.45µm filter, grounded in liquid nitrogen and then stored at -80 °C before nucleic acid extraction. Secondary metabolites were extracted from filtrates of 2 to 18 days old cultures medium. Three replications of each sample were analyzed.

Nucleic acid extraction

Rapid method of genomic DNA extraction (Lui *et al.*, 2000) was used for transformants screening by PCR. Large quantity genomic DNA was extracted by CTAB extraction method (Gardes & Bruns, 1993). The quality and quantity of DNA were estimated by measuring OD 260 nm / OD 280 nm and OD 260 nm respectively.

Total RNA was extracted by using the Tri-reagent (Euromedex France) DNA / RNA/ Protein extraction kit. The quality and quantity of RNA was checked by the OD 260 nm / OD 280 nm ratio and agarose gel electrophoresis according to standard protocols (Sambrook *et al.*, 1989).

Cloning of aolc35-6 gene in *A.westerdijkia* NRRL 3174

Degenerated primer pairs LC3 / LC5c (Bingle *et al.*, 1999) was used to identify *aolc35-6* gene. Table 1 list all the primers used in this study. Figure 2a marks position of specific primers LCS1 and LCS2 and degenerated primers ATR1 and ATR2 (designed at the most conserved regions **FTGQGAQ** and **AYSHHSM** of different *PKS* genes present in the

database) used to prolong the keto-synthase (KS) domain of *aolc35-6* gene by adopting degenerated primer based PCR strategy. The PCR products were cloned in pCR2.1-Topo plasmid and sequenced. Alignment of the sequenced fragments was performed to search for consensus.

PCR reaction and sequencing

PCR was performed with the *Taq* recombinant polymerase (Invitrogen, USA). Amplification was carried out in a 50 µl reaction mixture containing: 5 µl of *Taq* polymerase 10× buffer, 1·5 µl of 50 mM $MgCl_2$, 1 µl of dNTP 10 mM of each (Promega), 1 µM of each primer, 1·5 U of *Taq*, about 200 ng of DNA genomic, H_2O up to 50 µl. Reaction conditions were: 94 °C for 4 min, (94 °C for 45 s, 53 °C for 45 s and 72 °C for 1 min) × 30 cycles followed by an incubation at 72 °C for 10 min. The amplified products were examined by 1 % (w/v) agarose gel. The PCR products were cloned into pCR2.1-Topo vector (Invitrogen) according to the supplier's instructions. Sequencing of the fragments was performed by Genomexpress (Grenoble, France).

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://prodes.toulouse.inra.fr/multalin/multalin.html.

RT-PCR reaction

For RT-PCR, total RNA was treated with DNase I to remove DNA contamination. cDNA was synthesized from each sample with Advantage RT-for-PCR Kit (BD Biosciences) according to the supplier's manual. The expression of *aolc35-6* gene in *A. westerdijkiae* and *ao*Δ*msas* mutant (Bacha *et al.*, 2009b) was examined by using RT-PCR with two specific primers Aoat6-F and Aoat6-R designed from KS domain of the *aolc35-6* gene (Table 1). Further the expression of *aomsas* gene (Bacha *et al.*, 2009b) in *ao*Δ*lc35-6* mutant was examined by using specific primers AoLC35-2L and AoLC35-2R (Table 1). cDNA amplification were performed using Taq recombinant polymerase

(Invitrogen,USA). Beta tubulin was used as positive control using primers TubF and TubR (Table 1).

Plasmid construction and transformation of A. westerdijkiae

The *aolc35-6* gene was disrupted by inserting the *Escherichia coli* hygromycin B phosphotransferase gene (*hph*) flanked by *A. nidulans trpC* promoter and terminator sequences (Cullen *et al.*, 1987) from plasmid pID2.1 (Tang *et al.*, 1992). A modified form of PCR- mediated generation of gene disruption technique (Kuwayama *et al.*, 2002) was used to construct a *hph* cassette containing transformation vector (Fig. 1a). This technique is composed of three rounds of PCRs; in the first round, full length *hph* cassette, 5`- aolc35-6 containing *hph* tail and 3`- aolc35-6 containing *hph* tail is amplified; in the second round, 5`- aolc35-6 containing *hph* tail fragment is fused with the full length *hph* cassette to obtained *hph* cassette flanked by 5`- aolc35-6 fragment; and in the third round, *hph* cassette flanked by 5`- aolc35-6 fragment was fused with 3`- aolc35-6 containing *hph* tail fragment to obtained TopoLC*hph* vector. Different primers used during this approach are listed in table 1. The final fusion PCR product in which the *hph* cassette is flanked by *aolc35-6* fragments was cloned into pCR2.1-Topo plasmid (Invitrogen) generating the TopoLC*hph* plasmid (Fig. 1a). To construct the complementation vector TopoLC, PCR primers AoLc35-6disF and Aoat6-R were designed to amplify the full length aolc35-6 gene. This fragment was cloned into pCR2.1-Topo plasmid to construct TopoLC vector.

The *aolc35-6* gene inactivation and complementary mutant production were achieved by preparing protoplasts of the corresponding strains and transforming with TopoLC*hph* (Fig. 1b) and TopoLC respectively as previously described (O'Callaghan *et al.*, 2003). 40 mg/mL lysing enzyme (Sigma) was used for the preparation of protoplasts.

Screening of transformants

The *aolc35-6* disrupted mutants "*aoΔlc35-6*" and genetically complemented mutants *ao+lc35-6* were initially selected on YES medium (20 g/l of yeast extract, 1 M sucrose) supplemented with 150 µg/mL of hygromycin B. Transformant plates were incubated at room temperature for 24 h and then transferred to 30°C for 4 days. The positively screened transformants were further screened through a PCR, using *hph* gene specific

primers *hph*2F and *hph*2R and *aolc35-6* gene specific primer Aolc35-6disF (Table 1). Finally the disruption was confirmed in 3 clones through southern hybridization (Southern, 1975), in which total genomic DNA from both wild type *A. westerdijkiae*, *ao*Δ*lc35-6* and *ao+lc35-6* transformants were digested with restriction enzyme *Bal1*. This restriction enzyme was chosen because it cut only once in the 5′- flanking region of *ao*Δ*lc35-6* transformant (position 968 bp) but does not cut the *hph* cassette or 3′- flanking end of the *ao*Δ*lc35-6* (Fig. 1b). The digested DNA was then transferred to nylon membrane and probed with radioactively labelled DNA fragments of *aolc35-6* gene "lcp", amplified through primer pair Aoat6-F / Aoat6-R (probe position is shown in Figure 1 b).

Extraction of secondary metabolites

For secondary metabolites extraction, 30 mL filtrate sample of the culture medium was acidified with 200 µL of 12 N HCl, mixed with 30 mL chloroform and vigorously shaken for 10 min. The solvent phase was then decanted, dried under vacuum and re-dissolved in 0.5 mL Methanol. 20 µL of the sample was then further analysed by HPLC.

High-performance liquid-chromatography (HPLC) analysis

The HPLC apparatus consisted of a solvent delivery system, with both fluorescence (λ_{ex} = 332 nm; λ_{em} = 466 nm) and UV detectors (BIO-TEK, Milan, Italy). The analytical column used was a 150 x 4.6 mm Uptisphere 5 µm C18 ODB fitted with a guard column of 10 x 4 mm. The column temperature was 30 °C. Kroma 3000 (BIO-TEK) was the data acquisition system. Injections were done with an auto-injector (BIO-TEK, Milan, Italy) and the injection volume was 20 µL. The samples were analyzed by linear gradient elution using 0.2 % glacial acetic acid in 99.8 % water (v/v) (A) and 100 % acetonitrile (HPLC grade) (B). The crude extract was analyzed using a linear elution gradient over 45 min at a flow rate of 1 ml/min, starting from 10 to 50% solvent B over the first 30 min, continued by a linear gradient to 90 % of B in 5 min, followed by an isocratic flow of 90% solvent B for 8 min, and a return to initial conditions over the last 2 min of the run.
Secondary metabolites of *A. westerdijkiae* were detected by comparing the elution time and maximum absorption of UV with the standards (Sigma Aldrich France). All the standards were used at a concentration of 10 µg / mL. Isoasperlactone/ asperlactone

(wavelength λmax = 220 nm) released between 7 min and 9min, and PA (wavelength λmax = 227 nm) released at 11 min 50 sec.

Chemical complementation study of aoΔlc35-6 mutants

In two Erlenmeyer flask of 250 mL, containing 100 mL SAM medium, *aoΔlc35-6* mutants (density ~ 10^6 /mL) were inoculated and incubated at 25°C. After 72 hours 0.7 mM of 6- methylsalicylic acid (6-MSA) was added to one growing culture and 0.7 mM orsellinic acid was added to the other growing cultur and again incubated at 25°C without shaking. After 4 days from precursor addition secondary metabolites were extracted for HPLC analyses

Sequence accession number

The sequence obtained was deposited in Genbank under the accession number AY540951.

Results

Sequencing and characterization of *aolc35-6* gene in *A. westerdijkiae*

By using the degenerated primers LC3 / LC5c (Bingle *et al.*, 1999), a 0.7 kb ketosynthase (KS) domain fragment of a putative *PKS* gene "*aolc35-6*" has been cloned in *A. westerdijkiae* (Atoui *et al.*, 2006). Following the genome walking strategy, the degenerated primers ATR1 and ATR2 and specific primers LCS1 and LCS2 (table 1) in two consecutive PCRs, allowed the sequencing of an additional 1.29 kb fragment of *aolc35-6* gene (Fig. 2a).

The protein-protein blast search revealed that, *aolc35-6* has an identity of 60 % to the hybrid *PKS-NRPS* genes of *A. clavatus* (GeneBank protein ID: XP_001270445.1) and *A. niger* (GeneBank protein ID: XP_00139246.1) 57 % to *PKS-NRPS* of *Magnaporthe grisea* (GeneBank protein ID: CAG28798.1AJ704623) and 32 % to *msas* type *PKS* gene of *A. westerdijkiae* (GeneBank protein ID: AAS98200.1). Further a 300 bp fragment from the KS domain sequence of *aolc35*-6 gene was subjected to neighbour joining phylogenetic analysis against various genes of WA-type PKSs, MSAS type PKSs and hybrid PKS-NRPS clades. The phylogenetic tree showed that *aolc35-6* gene of *A. westerdijkiae* is clustered with hybrid PKS-NRPS genes clad (Fig. 2b).

Expression of *aolc35-6* gene and biosynthesis of secondary metabolites in *A. westerdijkiae*

To study the kinetic production of various secondary metabolites, *A. westerdijkiae* was grown in liquid synthetic medium (SAM). The fungus presented two exponential growth phases from day 3 to day 5 (early exponential growth phase) and day 6 to day 9 (within the late exponential growth phase) respectively, followed by a stationary phase from day 9 onward (Fig. 3a). PA, isoasperlactone and asperlactone were detected after day 4 in *A. westerdijkiae* liquid culture. Asperlactone and isoasperlactone productions abruptly increased and achieved their maximum levels at day 10 and 11 respectively. In contrary, the biosynthesis of PA remained comparatively low during the exponential growth phase of the fungus, thereafter it increased and reached its maximum level at day 12 of fungal growth. Then all the three metabolites entered into the reverse or negative log phase of productions (Fig. 3a). Two metabolites, 6-MSA and orsellinic acid were transitorily

produced in low quantity respectively on day 6 and day 8 of *A. westerdijkiae* growth (Fig. 3b).

Tracking the transcriptional signals of *aolc35-6* gene during different growth stages of *A. westerdijkiae* in synthetic medium (SAM), we observed that expression of the gene started from day 3 onwards, stayed constant at maximum level between day 5 and 8, and then stopped after day 11 (Fig. 3c).

Disruption of aolc35-6 gene in *A. westerdijkiae*

The *aolc35-6* gene in *A. westerdijkiae* was disrupted through TopoLC*hph* transformation vector by using protoplast based transformation technique (Fig. 1b). Following transformation, eighty putative *ao*Δ*lc35-6* transformants were screened through hygromycine containing CYA medium. In order to monitor integration of *hph* cassette in the genome of *A. westerdijkiae*, twenty one transformants were subsequently analyzed through two consecutive PCRs. Three out of the twenty one transformants responded positively to the PCR tests. Digested genomic DNA from these transformants and wild type *A. westerdijkiae* were subjected to southern hybridization. In the wild type *A. westerdijkiae* a signal corresponding to 1.7 kb was observed when its digested genomic DNA was probed with lcp probe (Fig. 4a). Probing of the *aolc35-6* disrupted mutants i.e. *ao*Δ*lc35-6a* and *ao*Δ*lc35-6b*, with lcp probe resulted into an expected signal of 4.1 kb (1.7 kb *aolc35-6*+2.4 kb *hph*) (Fig. 4a).

Both *ao*Δ*lc35-6* mutants and wild type *A. westerdijkiae* were inoculated in SAM medium and incubated for 10 days at 25°C. During their growth we observed no difference in fungal growth, sporulation, or pigment production. Tracking the HPLC profiles and UV spectra of secondary metabolites, we analyzed that disruption of *aolc35-6* gene not only abolish the biosynthesis of PA (wavelength λmax = 227 nm, released at 11 min 50 sec), but also isoasperlactone and asperlactone (wavelength λmax = 220 nm, released between 6 min and 9 min) (Fig. 4b). *ao*Δ*lc35-6* mutants also lost the capacity to accumulate the transitory production of 6-MSA and orsellinic acid which were detected in the wild type *A. westerdijkiae* (data not shown).

To confirm the role of *aolc35-6* gene, genetic complementation of *ao*Δ*lc35-6* mutants were performed. After several selection and screening tests (Fig.4a), the genetically

complemented mutant "*ao+lc35-6*" was inoculated in SAM medium and incubated for 10 days at 25°C for secondary metabolites production. HPLC profile and UV spectra of secondary metabolites revealed that reinsertion of *aolc35-6* gene restored the biosynthesis of all the missing metabolites including 6-MSA and orsellinic acid (Fig. 4b).

Correlative study of PA, isoasperlactone and asperlactone genes in *A. westerdijkiae*

To find out the relation between *aolc35-6* gene and the first asperlactone and isoasperlactone gene "*aomsas*" (Bacha *et al.*, 2009b) in *A. westerdijkiae*, we studied the expression of *aolc35-6* gene in *aomsas* disrupted mutant i.e. *ao*Δ*msas*, and the expression of *aomsas* gene in wild type *A. westerdijkiae* and in the *aolc35-6* gene disrupted mutant i.e. *ao*Δ*lc35-6*. No difference was observed in transcriptional signals of *aolc35-6* gene in the wild-type *A. westerdijkiae* and in *ao*Δ*msas* mutant (Fig. 3c). In contrary no transcriptional signals of *aomsas* gene was observed in *ao*Δ*lc35-6* mutant (Fig. 3c).

Orsellinic acid and 6-MSA are intermediates of PA and isoasperlactone / asperlactone respectively

Chemical complementation was performed by independent additions of 6-MSA and orsellinic acid to the growing cultures of *ao*Δlc35-6 mutants. Results show that incorporation of 6-MSA (0.7 mM) to the growing cultures of *aoΔlc35-6* mutants restored the biosynthesis of isoasperlatone and asperlactone but not PA (Fig. 5a). On the other hand *ao*Δlc35-6 mutants restart accumulation of PA when their growing cultures were incorporated with orsellinic acid (0.7 mM) (Fig. 5b).

Discussion

Based on molecular architecture of the already identified PKS genes, PKSs are previously classified into three distinct clades i.e. hybrid PKS-NRPS clad, WA type PKS clad and methylsalicylic acid synthase (MSAS) type PKS clad (Bingle *et al.*, 1999; Jing *et al.*, 2008; Nicholson *et al.*, 2001). Bingle *et al*. (1999), designed degenerated primers LC3 / LC5c to amplify Keto-Synthase (KS) domains of MSAS type fungal PKS genes (Lee *et al.*, 2001b; Varga *et al.*, 2003). We have previously used these degenerated primers and cloned a 700 bp KS domain fragment of a putative PKS gene i.e. *aolc35-6*, in *A. westerdijkiae* (Atoui *et al.*, 2006). After extension of the KS domain fragment of *aolc35-6* gene to a length of 1.999 kb (Fig. 2a), protein-protein blast search and neighbour joining phylogenetic analysis revealed that this gene is more close to the hybrid PKS-NRPS clad than WA type PKS or MSAS type PKS clades (Fig. 2a and 2b). Further to differentiate between PKS and hybrid PKS-NRPS genes certain amino acid patterns have been identified (Jing *et al.*, 2008; Moffitt *et al.*, 2003). Alignment study revealed that *aolc35-6* gene contain the most conserved characteristic pattern "VDTACSSS" of the KS domain of fungal and bacterial PKSs (Fig. 2a) (Atoui *et al.*, 2006). Moffitte *et al*. (2003) demonstrated that this characteristic conserved pattern has been replaced by "VQTACSTS" pattern in the bacterial PKS-NRPS genes. In contrary, alignment of fungal PKS and PKS-NRPS genes revealed that changing from fungal PKSs to fungal PKS-NRPSs no modification in the conserved pattern mentioned by Moffitte *et al*. (2003) occurs (Fig. 2a).

Transcriptional signals of *aolc35-6* could be correlated to the biosynthetic pattern of PA, isoasperlactone or asperlactone in *A. westerdijkiae* (Fig. 3).This statement was further confirmed when the *aolc35-6* knockout mutants i.e. *aoΔlc35-6a, aoΔlc35-6b and aoΔlc35-6c*, in *A. westerdijkiae* were found deficient in PA, isoasperlactone and asperlactone biosynthesis (Fig. 4b). Interestingly the mutants also lost the transitory biosynthesis of orsellinic acid and 6-MSA. Previously an MSAS type PKS gene "*aomsas*" has been characterized in *A. westerdijkiae* (Bacha *et al.*, 2009b). The *aomsas* knock out mutant "*ao*Δ*msas*" was found deficient in the production of isoasperlactone, asperlactone and 6-MSA. This means that *aolc35-6* could be the second gene important for isoasperlactone and asperlactone biosynthesis. Additionally we performed

experiments to establish relation between *aomsas* and *aolc35-6* genes. During these experiments the expression pattern of *aomsas* and aolc35-6 genes were analyzed during the growth of *aoΔlc35-6* and *aoΔmsas* mutant (Fig. 3c). *aolc35-6* gene produced similar transcriptional patterns both in wild type and in *aoΔmsas* mutant. But in contrary *aomsas* gene is no more expressing itself in *aoΔlc35-6* mutant. These observation suggest that product of *aolc35-6* could be important for the induction of *aomsas* gene expression in *A. westerdijkiae*. We further suggest that, there could be another unknown *aolc35-6* dependent PKS or related gene specifically important for PA biosynthesis.

Biochemically it has been previously demonstrated that orsellinic acid is an important intermediate of PA in *Penicillum cyclopium* (Axberg & Gatenbeck, 1975; Sekiguchi *et al.*, 1987). Further 6-MSA were found to be involved in the biosynthesis of an important polykeitde intermediate patuline in *Byssochlamys fulva* (Puel *et al.*, 2007). On one hand *A. westerdijkiae* can produce both PA and orsellinic acid but on the other hand this fungus is producing 6-MSA but not patuline (Fig. 3a and 3b). To confirm the role of orsellinic acid and to assign function to 6-MSA in *A. westerdijkiae*, independent incorporation of these compounds to separate growing cultures of *aoΔlc35-6* mutants (deficient in both orsellinic acid and 6-MSA) were performed. We confirmed that orsellinic acid is an important intermediate of PA (Fig. 5a). About 6-MSA, we demonstrated that this metabolite is not only important for patuline in *B. fulva* (Puel *et al.*, 2007) but also isoasperlactone and asperlactone in *A. westerdijkiae* (Fig.5b).

Based on some proposed biosynthetic pathways of PA (Axberg & Gatenbeck, 1975; Sekiguchi *et al.*, 1987) and isoasperlactone and asperlactone (James & Andrew, 1991), and our chemical complementation, *aolc35-6* gene disruption and expression studies, we proposed a hypothetic biosynthetic scheme could be biosynthesis of these metabolites (Fig. 6). This scheme could be composed of three pathways. The first pathway (Fig. 6i) could lead to the production of an unknown metabolite via the activity of *aolc35-6* gene. In the second biosynthetic pathway (Fig. 6ii) the product of *aolc35-6* gene could induce the expression of an MSAS type PKS gene i.e. *aomsas*, which could lead to the production of isoasperlactone and asperlactone, where 6-MSA was identified as an important intermediate. According to James & Andrew (1991) aspyrone and diepoxide are intermediates in the biosynthetic pathway of isoasperlactone and asperlactone. But in

case of *A. westerdijkiae*, we never detected any aspyrone and diepoxide; which could mean that this fungi is either a non-producer of these metabolites or these metabolites are never accumulated in detectable quantity. In the third biosynthetic pathway (Fig. 6iii), the product of *aolc35-6* gene could induces the expression of an unknown PKS gene, which could lead to the production of PA via orsellinic acid biosynthesis which identified in *A. westerdijkiae* as an important intermediate.

Although we have provided established avoidance about PA, isoasperlactone and asperlactone biosynthesis in *A. westerdijkiae*, but further experiments have to be performed for better understanding of different steps involved in the biosynthetic pathways of these important metabolites.

AKNOWLEDGMENTS

The authors are grateful to the Higher Education Commission of Pakistan for according PhD scholarship to Mr. Nafees Bacha.

References

1 Frisvad, J. C., Frank, J. M., Houbraken, J. A. M. P., Kuijpers, A. F. A., and Samson, R. A. (2004) New ochratoxin A producing species of *Aspergillus section Circumdati*, *Stud Mycol* **50**: 23–43.

2 Christensen, M., and Tuthill, D. E. (1985) Aspergillus: an overview In:. Samson RA, Pitt JI, eds. Advances in Pencillium and Aspergillus systematics: NY: Plenum Press.

3 Kozakiewicz, Z. (1989) Aspergillus species on stored products, *Mycol Pap* **161**: 1-188.

4 Antonia, G., Giancarlo, P., Michele, S., Filomena, E., Abdelhamid, A., Alan, D. W. D., and Giuseppina, M. (2008) Characterisation of a pks gene which is expressed during ochratoxin A production by *Aspergillus carbonarius*, *Int J Food Microbiol* **129**: 8-15.

5 Balcells, M., Canela, R., Coll, j., Sanchis, V., and Torres, M. (1995) Effect of fungal metabolites and some derivatives against *Tribolium castaneum* (Herbst) and *Nezara viridula* (L.), *Pestic sci* **45**: 319-323.

6 Torres, M., Balcells, M., Sala, N., Sanchis, V., and Canela, R. (1998) Bactericidal and fungicidal activity of *Aspergillus ochraceus* metabolites and some derivatives, *Pesticide Science* **53**: 9-14.

7 Atoui, A., Dao., P., Mathieu, F., and Lebrihi, A. (2006) Amplification and diversity analysis of Ketosynthase domains of putative polyketide synthase gene in *Aspergillus ochraceus* and *Aspergillus carbonarius* producers of ochratoxin A, *Mol Nutr Food Res* **50**: 448-493.

8 Mosbach, K. (1969) Zur Biosynthese roy Flechtenstoffen, Produkten einer symbiotischen Lebensgemeinschaft, *Angew Chem* **81**: 233-244.

9 Madhyastha, M. S., Marquardt, R. R., Masi, A., Borsa, J., and Frohlich, A. A. (1994) Comparison of toxicity of different mycotoxins to several species of bacteria and yeasts : use of *Bacillus brevis* in a disc diffusion assay, *J Food Port* **57**: 48-53.

10 Michito, T., Yoichi, I., and Hisae, M. (1996) Herbicide, *Japan Patent*: 08-053310.

11 Kang, S. W., and Kim, S. W. (2004) New antifungal activity of pinicillic acid against phytophthora species, *Biotechnol. Lett.* **26**: 695-698.

12 Rosenbrook, W. J., and Carney, R. E. (1970) New metabolite from an unidentified *Aspergillus species, Tetrahedron Letters* **22**: 1867-1870.

13 Balcells, M., Canela, R., Sala, N., Sanchis, V., and Torres, M. (1998) Bactericidal and fungicidal activity of *Aspergillus westerdijkiae* metabolites and some derivatives, *Pestic sci* **53**: 00009-00014.

14 Axberg, K., and Catenbeck, S. (1975) Intermediates in the penicillic acid biosynthesis in *Penicillium cyclopium*., *Acta Chem Scand B* **29**: 749-751.

15 Sekiguchi, J., Katayama, S., and Yamada, Y. (1987) 6-Methyl-1,2,4-Benzenetriol, a new intermediate in Penicillic acid biosynthesis in *Penicillum cyclopium*, *App Environ Microbiol* **53**: 1531-1535.

16 James, S., and Andrew, C. S. (1991) 17O NMR in biosynthetic studies: aspyrone, asperlactone and isoasperlactone, metabolites of *Aspergillus melleus*, *J Chem Soc., Chem Commun*: 1106-1108.

17 Metz, J. G., Roessler, P., Facciotti, D., Levering, C., Dittrich, F., Lassner, M., et al. (2001) Production of polyunsaturated fatty acids by polyketide synthase in both prokaryotes and eukaryotes, *Science* **293**: 290-293.

18 Niermanm, W. C., Pain, A., Anderson, M. J., Wortman, J. R., Kim, H. S., Arroyo, J., et al. (2005) Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*, *Nature* **438**: 1151-1156.

19 Sebastian, B., Julia, S., Kirstin, S., Corinna, L., Axel, A. B., and Christian, H. (2007) Genomics-driven discovery of PKS-NRPS hybrid metabolites from *Aspergillus nidulans*, *Nature chem biol* **3**: 213-217.

20 Du, L., and Shen, B. (2001) Biosynthesis of hybrid peptide-polyketide natural products, *Curr. Opin. Drug Discovery Dev* **4**: 215-228.

21 Song, Z., Cox, R. J., Lazarus, C., and Simpson, M. T. J. (2004) Biosynthesis of Fusarin C in *Fusarium moniliforme* and *Fusarium venenatum*, *Chem Bio Chem* **5**: 1196-1203.

22 Sims, J. W., Fillmore, P., Warner, D. D., and Schmidt, E. W. (2005) Equisetin biosynthesis in *Fusarium heterosporum*, *Chem. Commun*: 186-188.

23 Eley, K. L., Halo, L. M., Song, Z., Powles, H., Cox, R. J., Bailey, A. M., et al. (2007) Biosynthesis of the 2-Pyridone tenellin in the Insect pathogenic fungus *Beauveria bassiana*, *Chem Bio Chem* **8**: 289-297.

24 Bingle, L. E., Simpson, T. J., and Lazarus, C. M. (1999) Ketosynthase domain probes identify two subclasses of fungal polyketide synthase genes, *Fungal Genet Biol* **26**: 209-223.

25 Nicholson, T. P., Rudd, B. A. M., Dawson, M., Lazarus, C. M., Simpson, T. J., and Cox, R. J. (2001) Design and utility of oligonucleotide gene probes for fungal polyketide synthases, *Chem Biol* **8**: 157-178.

26 de Crecy-Lagard, V., Saurin, W., Thibaut, D., Gil, p., Naudin, l., Crouzet, J., and Blanc, V. (1997) Streptogramin B biosynthesis in *Streptomyces pristinaespiralis* and *Streptomyces virginiae*: molecular characterization of the last structural peptide synthetase gene. , *Antimicrob. Agents Chemother.* **41**: 1910-1915.

27 Lui, D., Caloe, C., Biard, R., and Pedersen, J. (2000) Rapid mini preparation of fungal DNA for PCR, *J Clin Microboil* **38**: 471.

28 Gardes, M., and Bruns, T. D. (1993) TS primers with enhanced specificity for Basidiomycetes-applicatin to the identification of mycorrhizae and rusts, *Mol Ecol* **2**: 113-118.

29 Sambrook, J., Fritsch, E. F., and Maniatis , T. (eds) (1989) Molecular cloning: a laboratory Manual. Cold Spring Harbor: Cold Spring Harbor laboratory press.

30 Bacha, N., Dao, H. P., Atoui, A., Mathieu, F., O'Callaghan, J., Puel, O., et al. (2009) Cloning and characterization of novel methylsalicylic acid synthase gene involved in the biosynthesis of isoasperlactone and asperlactone in Aspergillus westerdijkiae, *Fungal Genet Biol*: in press.

31 Cullen, D., Leong, S. A., Wilson, L. J., and Henner, D. J. (1987) Transformation of *Aspergillus nidulans* with the hygromycin-resistance gene, *hph*, *Gene* **57**: 21-26.

32 Tang, C. M., Cohen, J., and Holden, D. W. (1992) An *Aspergillus fumigatus* alkaline protease mutant constructed by gene disruption is deficient in extracellular elastase activity, *Molecular Microbiol* **6**: 1663-1671.

33 Kuwayama, H., Shinji, O., Takahiro, M., Mariko, K., Hideko, U., and Yoshimasa, T. (2002) PCR-mediated generation of a gene disruption construct without the use of DNA ligase and plasmid vectors, *Nucleic. Acids. Res.* **30**: 2e2.

34 O'Callaghan, J., Caddick, M. X., and Dobson, A. D. W. (2003) A polyketide synthase gene required for ochratoxin A biosynthesis in *Aspergillus ochraceus*, *Microbiol* **149**: 3485-3491.

35 Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis, *J Mol Biol* **98**: 503-517.

36 Jing, Z., Ning, Y., and Runying, Z. (2008) Phylogenetic analysis of type I polyketide synthase and nonribosomal peptide synthetase genes in *Antarctic sediment*, *Extremophiles* **12**: 97-105.

37 Lee, T., Yun, S. H., Hodge, K. T., Humber, R. A., Krasnoff, S. B., Turgeon, G. B., et al. (2001) Polyketide synthase genes in insect- and nematode-associated fungi, *Appl Microbiol Biotechnol* **56**: 181-187.

38 Varga, J., Rigo, K., Kocsube, S., Farkas, B., and Pal, K. (2003) Diversity of polyketide synthase gene sequences in *Aspergillus* species, *Res Microbiol* **154**: 593-600.

39 Moffitt, M. C., Neilan, B. A., and (2003) Evolutionary affiliations within the superfamily of ketosynthases reflect complex pathway associations, *J Mol Evol* **56**: 446-457.

40 Axberg, K., and Gatenbeck, S. (1975) Intermediates in the penicillic acid biosynthesis in Penicillium cyclopium, *Acta Chem Scand Ser* **29**: 749-751.

41 Puel, O., S.Tadrist, Delafoege, M., Oswald, I. P., and Lebrihi, A. (2007) The inability of *Byssochlamys fulva* to produce patulin is related to absence of 6-methylsalicylic acid synthase and isoepoxydon dehydrogenase genes, *Int J Food Microbiol* **115**: 131-139.

Table 1. List of the oligonucleotide primers

Primers used in gene walking approach	
Primer Name	**Sequence (5'-3')**
LC3	GCNGARCAR ATGGAYCCNCA
LC5c	GTNGANGTNGCRTGNGCYTC
ATR1	YTG5GC5CCYTG5CC5GTDAA
ATR2	CATRTGRTGIGARTGRTAIGC
LCS1	CGGCCGCAATTCTTCGAA
LCS2	GCCATGCACAGCCACGCG
Primers used in fusion PCR[9] , post transformation analysis and RT-PCR	
106- Aoat6-R	CGTCCTGGAAGACAAGGTGT
3- AoLc35-6disF	AAGCTCTAGAATCCGGTGGCCTG
4- AoLC6*hph*R	<ATCCCCGGGTACCGAGCTCGAATTC>< GGCTCGACGAACTGAACC>
5- AoLC6*hph*F	<TCGACCTGCAGGCATGCAAGCTGGC><AACAGCTTTGGATTCGGT>
1- *hph*1F	GAATTCGAGCTCGGTACCCGGGGAT
2- *hph*1R	GCCAGCTTGCATGCCTGCAGGTCGA
Aoat6-F	ACGTGGTCGCTTAAAGAGGA
*hph*2F	CGGGGGCAATGAGATATGAAAAAG
*hph*2R	GAACCCGCTCGTCTGGCTAAG
TubF	CTCGAGCGTATGAACGTCTAC
TubR	AAACCCTGGAGGCAGTCGC
AoLC35-2R	CTGGAGGATCTCGCTGATGT
AoLC35-2L	TTTGATCGACCATTGTGTGC

[9] The two Primers i.e. 4 and 5, are designed according to the procedure described by Kuwayama *et al*, 2002.

[10] Each number indicate a primer code used in figure 4a

Figure legends and figures

Fig. 1.

Transformation vector construction and disruption of *aolc35-6* gene: (a) Modified form of PCR- mediated generation of gene disruption technique (Kuwayama *et al.*, 2002) was used to construct TopLC*hph* vector. Arrows marked by numbers indicate different primers. The blue colour tilted lines behind primers 4 and 5 indicate *hph* tils (b) Protoplasts of *A. westerdijkiae* were prepared and *aolc35-6* gene was disrupted using TopoLC*hph* vector to obtain *aoΔlc35-6* mutants. Different colours on the *aoΔlc35-6* indicate different functional domains i.e. *Escherichia coli* hygromycin B phosphotransferase gene, β-ketoacyl synthase (KS), acyltransferase (AT). The small red line below *aoΔlc35-6* indicates position of radioactively labelled probe lcp: *aolc35-6* gene specific probe amplified by using primer pair Aoat6F /Aoat6R. Position of the restriction sites of *Bal*1 is indicated by triangle.

Fig. 2.

Characterization of aolc35-6 gene in *A. westerdijkiae*: (a) Alignment of the deduced amino acid sequence of *A. westerdijkiae aolc35-6* gene with other hybrid PKS-NRPS gene of; *A. clavatus* (GeneBank protein ID: XP_001270445.1), *A. niger* (GeneBank protein ID: XP_00139246.1) and *Magnaporthe grisea* (GeneBank protein ID: CAG28798.1AJ704623), and PKSs gene of; *A. westerdijkiae* (GeneBank protein ID: AAS98200.1), *Gibberella fujikuroi* (GeneBank protein ID: CAC44633.1), *Botryotinia fuckeliana* (GeneBank protein ID: AAR90241.1) and *Aspergillus terreus* (GeneBank protein ID: Q9Y8A5). Rectangular box indicates the conserved pattern mentioned by Moffitte *et al*. (2003) which is modifications in bacterial PKS-NRPS genes. Flash arrows indicate positions of different specific and degenerated primers (table 1) used in gene walking experiment. (b) Neighbour joining phylogenetic tree of the KS domain sequences of fungal hybrid PKSs / NRPSs and PKSs. Bootstrap values above 50 are shown on the branches. Different clusters are indicated on the right. The accession numbers of genes are indicated in the brackets in front of organism name.

Fig. 3.

Kinetic production of secondary metabolites and expression of *aolc35-6* gene in *A. westerdijkiae*: (a) Kinetic production of PA, isoasperlactone, asperlactone, 6-MSA and orsellinic acid in a growing culture of *A. westerdijkiae* at 25°C in synthetic medium during a time course of 2-18 days. (b) Kinetic production of 6-MSA and orsellinic acid in a growing culture of *A. westerdijkiae* at 25°C in synthetic medium during a time course of 2-18 days (the volume obtained is multiplied with 10). (c) Profile of *aolc35-6* gene expression in wild-type *A. westerdijkiae* (first panel), *aolc35-6* gene expression in *ao*Δ*msas* mutant (second panel) (Bacha *et al.*, 2009b), *aomsas* gene expression in wild-type *A. westerdijkiae* (third panel), by RT-PCR. Beta tubuline was used as loading control (bottom panel).

Fig. 4.

Screening of the transformants and secondary metabolites production: (a) Genomic DNA of wild type *A. westerdijkiae*, *ao*Δ*lc35-6* and ao+lc35-6 transformants were digested with *Bal*1 restriction enzyme and probed with radioactively labelled *aolc35-6* fragment lcp. In the wild-type a 1.7 kb band was detected, while in the transformants *ao*Δ*lc35-6a*, *ao*Δ*lc35-6b* and *ao*Δ*lc35-6c* the 1.7 kb band is replaced by the expected 4.1 kb band. In the genetically complemented mutant *ao+lc35-6* the 4.1 kb band is replaced by the wild-type 1.7 kb band. (b) Secondary metabolites were extracted from 10 days old cultures of wild-type, *ao*Δ*lc35-6* and *ao+lc35-6* strains of *A. westerdijkiae*. HPLC traces of PA (eluted at 12 min), isoasperlactone (eluted at 6 min), asperlactone (eluted at 9 min) in the wild-type *A. westerdijkiae* were compared *ao*Δ*lc35-6* and *ao+lc35-6* transformants

Fig.5.

Chemical complementation study of *ao*Δ*lc35-6* mutants: (a) HPLC traces of secondary metabolites eluted from the culture medium of *ao*Δ*lc35-6* mutants incorporated with 0.7 mM of 6-MSA. (b) HPLC traces of secondary metabolites eluted from the culture medium of *ao*Δ*lc35-6* mutants incorporated with 0.7 mM of orsellinic acid.

Fig. 6.

Proposed biosynthetic scheme of PA, isoasperlactone and asperlactone: Based on the study of Sekiguchi *et al*, (1987) and James & Andrew, (1991), we proposed a hypothetical biosynthetic scheme of PA, isoasperlactone and asperlactone biosynthesis. The doted arrow lines indicate the biosynthetic route with unknown intermediates. The dashed arrow lines indicate possible biosynthetic path. Curved flash arrows indicate activities of different genes.

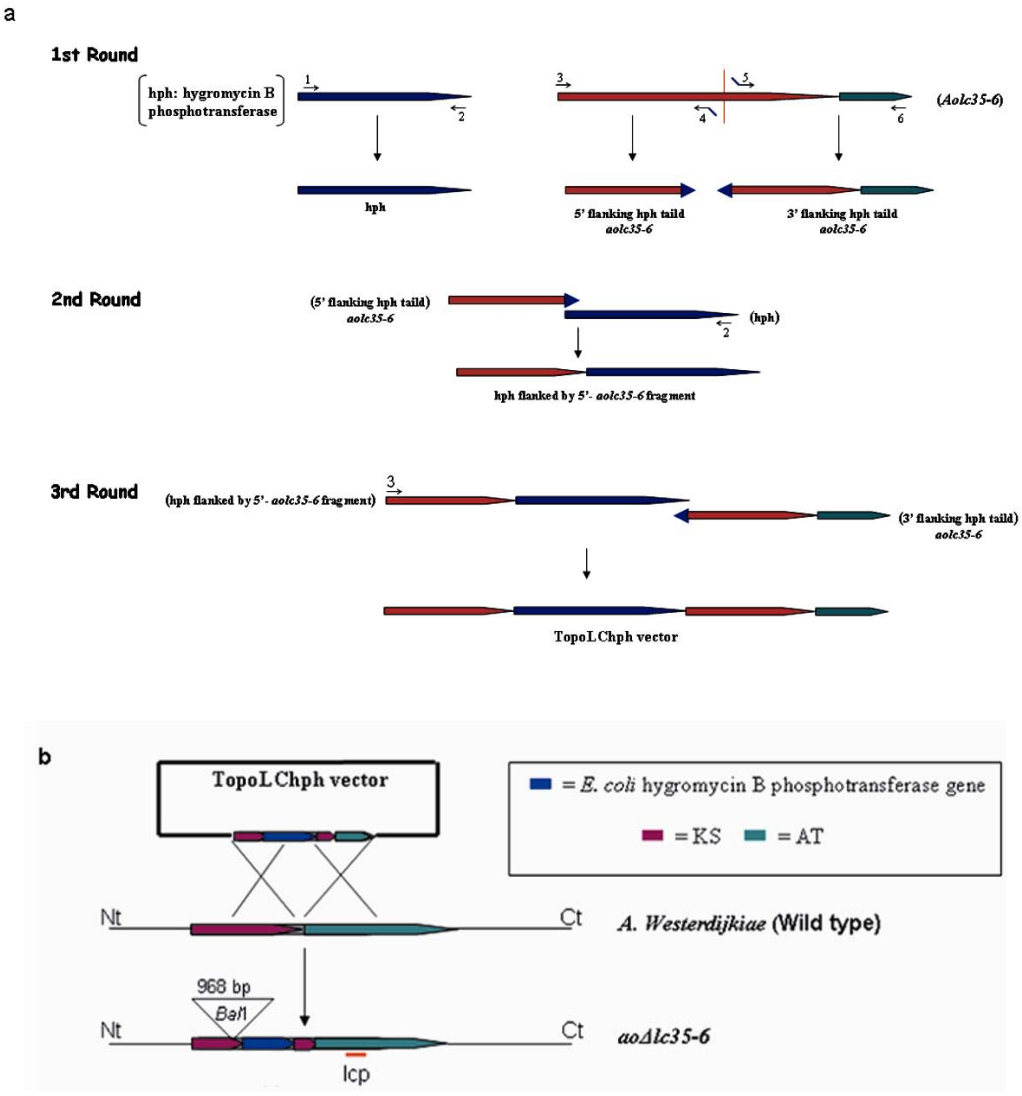

a
1st Round
hph: hygromycin B phosphotransferase
1
2
3
4
5
6
(Aolc35-6)
hph
5' flanking hph taild aolc35-6
3' flanking hph taild aolc35-6
2nd Round
(5' flanking hph taild) aolc35-6
(hph)
hph flanked by 5'- aolc35-6 fragment
3rd Round
(hph flanked by 5'- aolc35-6 fragment)
(3' flanking hph taild) aolc35-6
TopoLChph vector
b
TopoLChph vector
= E. coli hygromycin B phosphotransferase gene
= KS
= AT
Nt
Ct
A. Westerdijkiae (Wild type)
968 bp
BalI
Nt
Ct
aoΔlc35-6
lcp

Figure 1

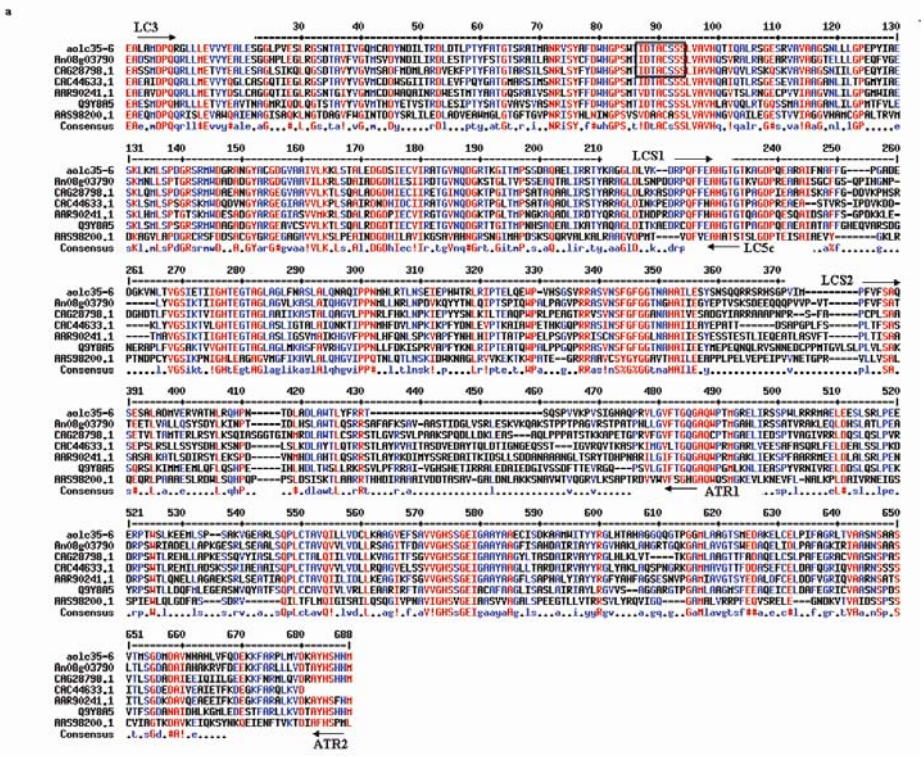

a
LC3
LCS1
LCS2
LCSc
ATR1
ATR2
aolc35-6
An08g03790
CAG28798.1
CAC44633.1
AAR90241.1
Q9YBA5
AAS98200.1
Consensus
b
aolc35-6
A . clavatus (4702558)
A . niger (An08g03790)
M. grisea (AJ704623)
A . clavatus (ACLA-078660)
G. fujikuroi (AJ315370)
B. Fuckeliana (AY495610)
P tritici-repentis (XM_0019342)
A. terreus (Q9Y8A5)
P. citrinum (AB072893)
A. Westerdijkiae (AY540947)
P. patulum (P22367)
Hybrid PKS / NRPS
WA-type PKSs
MSAS-type PKSs
100
79
81
0.1

Figure 2

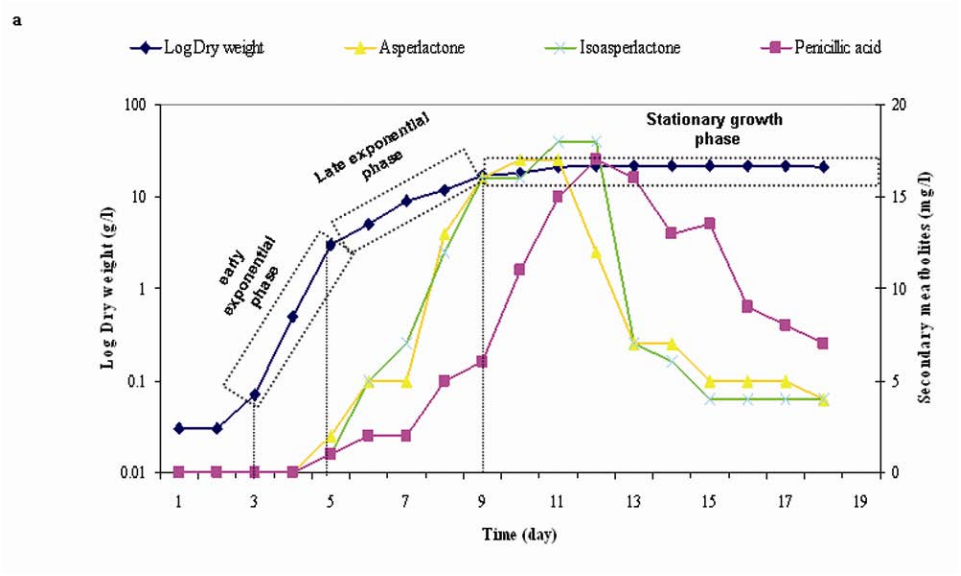

a
Log Dry weight
Asperlactone
Isoasperlactone
Penicillic acid
Stationary growth phase
Late exponential phase
early exponential phase
Log Dry weight (g/l)
Secondary metabolites (mg/l)
Time (day)
b
6-MSA
Orsellinic acid
secondary metabolite x 10 (mG/L)
Time (days)
c
d3
d4
d5
d6
d7
d8
d9
d10
d11
aolc35-6 in wild-type A. westerdijkiae
aolc35-6 in aoΔmsas
aomsas in wild-type A. westerdijkiae
aomsas in aoΔlc35-6
Beta tubulin

Figure 3

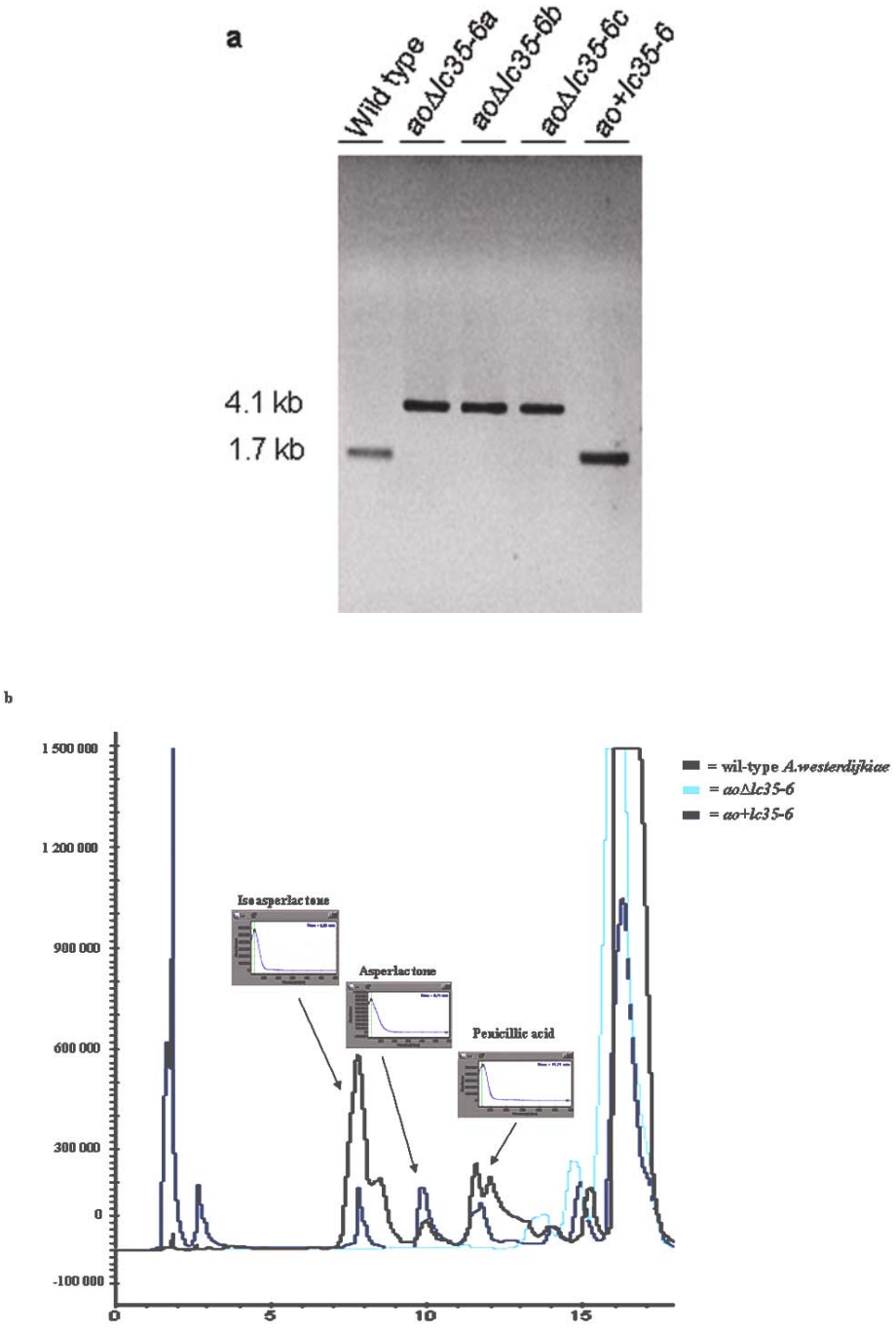

a
Wild type
aoΔlc35-6a
aoΔlc35-6b
aoΔlc35-6c
ao+lc35-6
4.1 kb
1.7 kb
b
1 500 000
1 200 000
900 000
600 000
300 000
0
-100 000
0
5
10
15
Isoasperlactone
Asperlactone
Penicillic acid
= wil-type A.westerdijkiae
= aoΔlc35-6
= ao+lc35-6

Figure 4

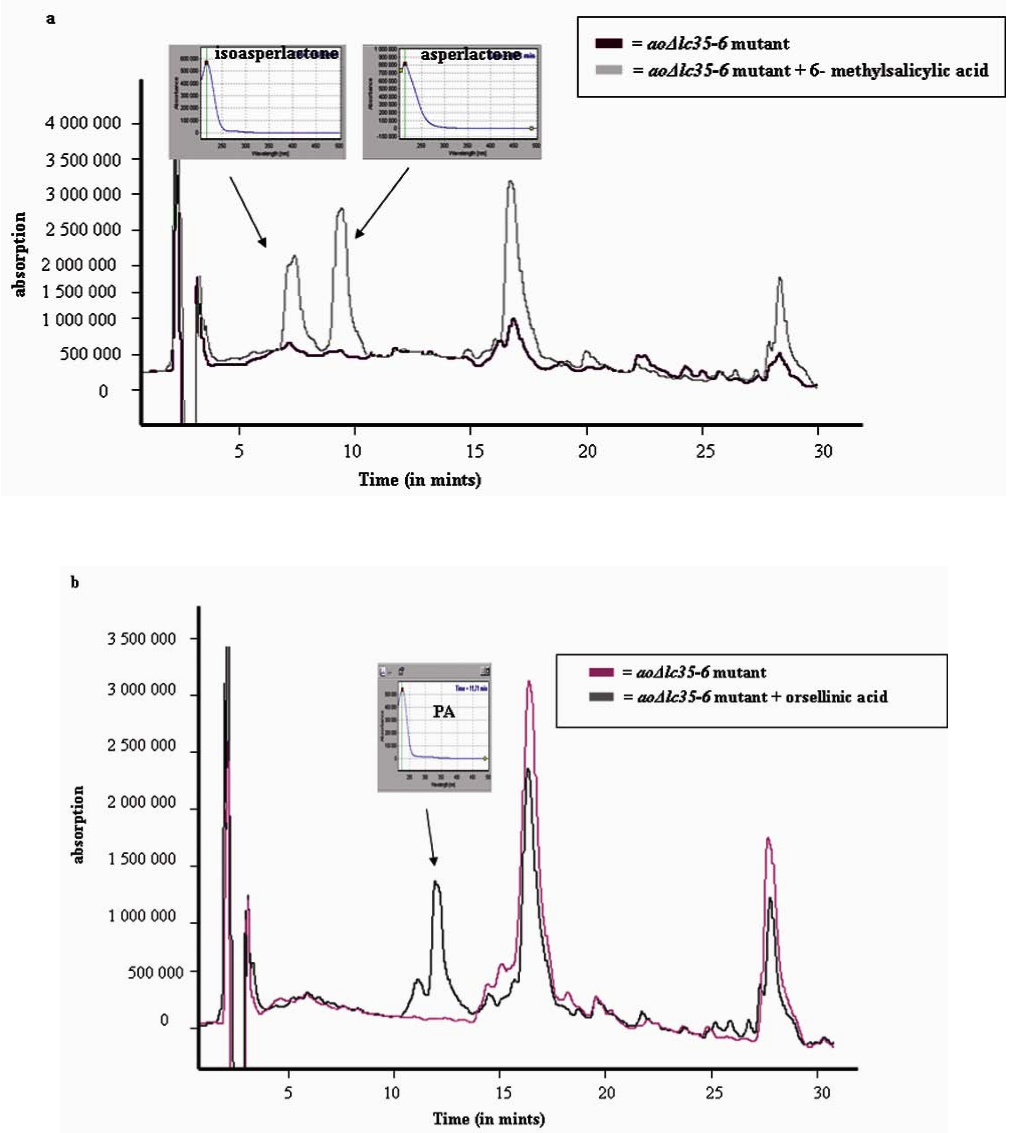

a
isoasperlactone
asperlactone
= aoΔlc35-6 mutant
= aoΔlc35-6 mutant + 6- methylsalicylic acid
absorption
4 000 000
3 500 000
3 000 000
2 500 000
2 000 000
1 500 000
1 000 000
500 000
0
5
10
15
20
25
30
Time (in mints)
b
PA
= aoΔlc35-6 mutant
= aoΔlc35-6 mutant + orsellinic acid
absorption
3 500 000
3 000 000
2 500 000
2 000 000
1 500 000
1 000 000
500 000
0
5
10
15
20
25
30
Time (in mints)

Figure 5

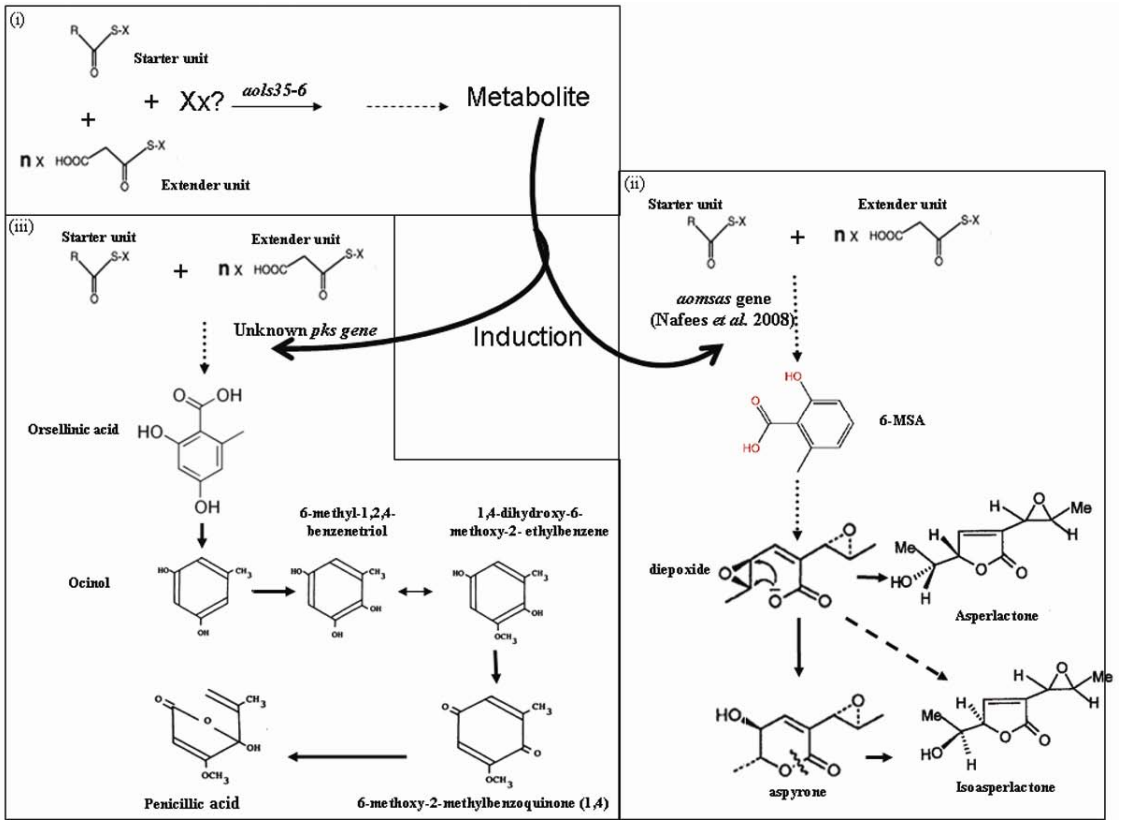

(i)
Starter unit
+ Xx?
aols35-6
Metabolite
+
n X HOOC
Extender unit
(ii)
Starter unit
Extender unit
n X HOOC
aomsas gene
(Nafees et al. 2008)
6-MSA
diepoxide
Asperlactone
aspyrone
Isoasperlactone
(iii)
Starter unit
Extender unit
n X HOOC
Unknown pks gene
Induction
Orsellinic acid
6-methyl-1,2,4-benzenetriol
1,4-dihydroxy-6-methoxy-2- ethylbenzene
Ocinol
Penicillic acid
6-methoxy-2-methylbenzoquinone (1,4)

Figure 6

General discussion and perspectives

4- General discussion and Perspectives

4.1 General discussion

In fungi polyketides are the largest group of metabolites occurring in their greatest number and variety especially in *Ascomycetes* and related "imperfect" fungi including *Aspergillus westerdijkiae*. *A. westerdijkiae,* which is recently dismembered from *A. ochraceus* (Frisvad *et al.*, 2004), is the principal producer of several important polyketide metabolites including ochratoxin A, mellein, hydroxymellein, penicillic acid, asperlactone and isoasperlactone (Atoui *et al.*, 2006; Gaucher and Shepherd, 1968). During the kinetic production of secondary metabolites study, we observed that this fungus presented two principal growth phases. The first one corresponded to the exponential phase starting from day 3 and end-up at day 9 of *A. westerdijkiae* growth, while the second one corresponded to the stationary phase starting after the exponential growth phase. The biosynthesis of most polyketide metabolites started from the early exponential growth phase; attained a certain maximum limit up to the early stationary growth phase of *A. westerdijkiae* and then their accumulation decreased to a certain level over the wrest of the growth. These observations suggested that the biosynthesis of polyketide secondary metabolites was associated with late growth and development of *A. westerdijkiae*. This fact has also been observed previously, where the biosynthesis of various secondary metabolites in *A. nidulans* and *Streptomyces* were found to be associated with growth, development or sporulation processes (Bu'Lock et al., 1968; Calvo et al., 2001; Calvo et al., 2002; Hopwood, 1988). We have further analyzed that two compounds 6- methylsalicylic acid (6-MSA) and orsellinic acid are transitorily produced in very low quantity between day 6 and day 8 of fungal growth. 6-MSA has been previously proposed to be the intermediate of two polyketide metabolites i.e. patuline in *P. patulum* (Beck *et al.*, 1990) avilamycine in *S. viridochromogenes* (Weitnauer *et al.*, 2001). But none neither patuline nor avilamycine has ever been detected in the culture of *A. westerdijkiae.* On the other hand orsellinic acid has been proposed to be an important intermediate of penicillic acid. We correlated the transitory biosynthesis of 6-MSA and orsellinic acid to their intermediate nature (Axberg and

Catenbeck, 1975; Beck *et al.,* 1990) and assume that these metabolites could readily be converted to certain polyketide end products like penicillic acid, asperlactone and isoasperlactone.

Recently in our laboratory Atoui *et al,* (2006), identified 9 different polyketide synthase (PKS) genes based on the conserved ketosynthase (KS) domain including *aoks1, aols35-12, aomsas* and *aolc35-6*. We adopted the degenerated primer based genome walking and RACE-PCR techniques to prolong KS domains of *aoks1*, *aolc35-12*, *aomsas*, and *aolc35-6* to a length of 2 kb, 3.22 kb, 5.298 kb and 1.999 kb respectively. Homology search distributed these genes into four different clusters. *aoks1* presented a high degree of similarity with lovastatine biosynthesis gene "*lovb*" in *A. terreus* (Hendrickson et al., 1999) and compactin biosynthesis gene "*mlcA*" in *Penicillium citrinum* (Abe et al., 2002), *aolc35-12* presented a high degree of similarity with OTA-PKS genes in *A. ochraceus* and *A. niger* (O'callaghan *et al*., 2003), *aomsas* presented high degree of similarity with different methylsalicylic acid synthase genes found in *Byssochlamys nivea, P. patulum, A. terreus* and *Streptomyces viridochromogenes* while *aolc35-6* was found very similar to different hybrid *PKS-NRPS* genes of *A. clavatus, A. niger* and *Magnaporthe grisea*.

To understand the expression patterns of these genes over the growth of *A. westerdijkiae* reverse transcription PCR were performed. Through this experiment we have analyzed that the transcriptional signals of most of these genes are distributed within the exponential growth phase of the fungi. By comparing the biosynthetic patterns of various polyketide metabolites with the transcriptional patterns of target genes, we hypothetically associated *aoks1* and *aolc35-12* with OTA biosynthesis while *aomsas*, and *aolc35-6* with penicillic acid, isoasperlactone and asperlactone biosynthesis.

Insertional inactivation was followed to generate *aoks1* and *aolc35-12* genes disrupted mutants *ao*Δ*ks1* and *ao*Δ*lc35-12* respectively. Both these mutants were found deficient in the biosynthesis of ochratoxin A, but were still producing mellein and hydroxymellein. Based on the structural similarity with OTA, mellein and hydroxylmellein were previously proposed to be an intermediate in the biosynthesis of OTA (Huff & Hamilton,

1979). Recently this hypothesis has been dissented by Harris and Mantle (2001) using labelled precursors of OTA. They found no evidence for the intermediate role of mellein in OTA biosynthetic pathway. Based on our observation we proposed that *aoks1* and *aolc35-12* are two PKS gene involved in the biosynthesis of OTA. We further support the hypothesis of Harris and Mantle (2001) and proposed that OTA is biosynthesized independently of mellein. To date, only three cases have been reported that involve two different fungal PKSs essential for a single polyketide: a set of two unusual type I multifunctional PKSs for the biosynthesis of lovastatin and compactin in *A. terreus* and *P. citrinum* respectively (Abe et al., 2002; Hendrickson et al., 1999; Kennedy et al., 1999) and two PKS have been reported to be involved in the biosysnthesis of the mycotoxins zearaleone in *Gibberella* zea (Kim et al., 20050 and T toxin in *Cochliobolus heterosphorus* (Baker et al., 2006).

It has been recently demonstrated that OTA-PKS gene in *A. carbonarius* is deferentially expressed in OTA permissive condition when compared with OTA suppressive condition (Antonia *et al.,* 2009). We have studied the deferential expression of *aoks1* and *aolc35-12* in *ao*Δ*ks1* and *ao*Δ*lc35-12* (OTA suppressive conditions) and compared it with the expression of these genes in wild-type *A. westerdijkiae* (OTA permissive conditions). Interestingly we have observed that in *ao*Δ*lc35-12* mutant *aoks1* gene has lost its transcriptional signals. Therefore we conclude that *aolc35-12* is a PKS gene that is not directly involve in the biosynthetic pathway of OTA but diferentially controls the expression of OTA biosynthetic pathway gene *aoks1*.

Disruption of *aomsas* gene abolished the biosynthesis of two end product polyketides “isoasperlatone and asperlactone” and one intermediate nature compound “6-methylsalicylic acid (6-MSA)”. Genetic complementation confirmed that *aomsas* is important for the biosynthesis of isoasperlactone, asperlactone and 6-MSA. It has been previously demonstrated that diepoxide and aspyrone are intermediates in the biosynthetic pathway of isoasperlactone and asperlactone in *A. melleus* (James & Andrew, 1991). These authors further stated that asperlactone is directly biosynthesized from the diepoxide, while in the biosynthesis isoasperlactone the diepoxide is first converted to aspyrone and then aspyrone to isoasperlactone. They also suggested an

alternate rout for the biosynthesis isoasperlactone without the involvement of aspyrone. We confirmed the observation of James & Andrew, (1991), by performing chemical complementation of the asperlactone / isoasperlactone deficient mutant i.e. *ao*Δ*msas*, through incorporation of 6-MSA, diepoxide and aspyrone. We have clearly demonstrated that, these products are intermediates in the biosynthetic pathway of asperlactone and isoasperlactone. Aspyrone and diepoxide are never observed in the culture medium of wild type *A. westerdijkiae*. This could probably be due to a quick conversion of these compounds to their final products (asperlactone and isoasperlactone), avoiding an intracellular accumulation and extracellular excretion.

Based on our observations we proposed a scheme for the biosynthesis of isoasperlactone and asperlactone, in which during the early biosynthetic steps *aomsas* gene induces the production of 6-MSA, which could then be converted to diepoxide. The diepoxide could then follow two routes, one that leads directly to the production of asperlactone while the second leads to the production of isoasperlactone via aspyrone pathway. We further proposed that diepoxide and aspyrone could be enter-convertible compounds which could lead to the production of both asperlactone and isoasperlactone. Our observations were in accordance to the previous findings, suggesting that lactonic metabolites asperlactone and isoasperlactone could be derived from a common biosynthetic precursor, a diepoxy intermediate (Sorensen and Simpson, 1986).

aolc35-6 gene was associated with hybrib PKS-NRPS based on alignment of its amino acid sequence in the data base. Inactivation of this hybrid PKS-NRPS gene in *A. westerdijkiae* revealed that this gene is involved in the biosynthesis of three lactonic metabolites penicillic acid, isoasperlactone and asperlactone and two other metabolites orsellinic acid and 6-MSA. It has been previously demonstrated that orsellinic acid is an intermediate of penicillic acid (Axberg and Catenbeck, 1975) and 6-MSA is an intermediate of patulin (Puel *et al.*, 2007). In *A. westerdijkiae* culture penicillic acid was present through in all growth stages while patuline was never observed in what ever incubation time. Chemical complementation studies by the addition of either orsellinic acid or 6-MSA to the growing cultures of the mutant *ao*Δ*lc35-6* restored the production of penicillic acid and isoasperlactone / asperlactone respectively. These results suggest

that *aolc35-6* is a hybrid PKS-NRPS gene equally important for the biosynthesis of penicillic acid, isoasperlactone and asperlactone. We further studied the impact of the two isoasperlactone / asperlactone genes "*aomsas*" and "*aolc35-6*" on each other. We have observed through diferential expression study of these genes that, *aolc35-6* code for certain precursor / intermediate differentially controlling the biosynthetic pathway of penicillic acid and isoasperlactone / asperlactone.

4.2 Perspectives

Findings of the present work permit us to project the following different perspectives;

- **Characterization of other PKS genes already cloned in *A. westerdijkiae***

In total 9 PKS genes are identified in *A. westerdijkiae* (Atoui et al., 2006), but only 4 of these genes have been functionally characterized. Characterization of the remaining 5 PKS genes through insertional inactivation will probably explore new aspects of the biosynthesis of different polyketides in *A. westerdijkiae*.

- **Cloning of other OTA biosynthesis genes in *A. westerdijkiae***

From the two OTA-PKS gene i.e. *aoks1* and *aolc35-12*, we characterized in *A. westerdijkiae* we observed that *aolc35-12* could diferentially control the biosynthetic genes of OTA. Hence suppression subtractive hybridization method could be useful to understand how these two genes are organized and to identify other OTA genes in this fungus.

- **Cloning of penicillic acid, isoasperlactone and asperlactone biosynthesis genes in *A. westerdijkiae***

The hybrid PKS-NRPS gene has been found to diferentially control the biosynthetic genes of isoasperlactone and asperlactone (*aomsas*) and penicillic acid (non-identified).

Suppression subtractive hybridization method could be useful for the identification of new genes that could play important roles in the biosynthesis of penicillic acid isoasperlactone and asperlactone biosynthesis. The identification of these genes can help us understanding the biosynthetic mechanisms of these metabolites in *A. westerdijkiae*.

- **Construction of genome bank of *A. westerdijkiae***

The different PKS genes identified and characterized in *A. westerdijkiae* could serve as a source for designing probes that could be used for the screening of genomic DNA bank of this fungus. This bank could help us in the identification of different polyketide biosynthesis gene clusters.

References

References

Abarca, M. L., Bragulat, M. R., Castella, G. & Cabanes, F. J. (1994). Ochratoxin A production by strains of *Aspergillus niger* var. niger. *Appl Environ Microbiol* **60**, 2650-2652.

Abe, Y., Suzuki, T., Ono, C., Iwamoto, K., Hosobuchi, M. & Yoshikawa, H. (2002). Molecular cloning and characterization of an ML-236B (compactin) biosynthetic gene cluster in *Penicillium citrinum*. *Mol Genet Genomics* **267**, 636-646.

Abell, C., Doddrell, D. M., Garson, M. J., Laue, E. D. & Staunton, J. (1983). Biosynthesis of the polyketide mellein from CD3CO2H and 13CD3CO2H in Aspergillus melleus: detection of deuterium by 2H n.m.r. and edited 13C n.m.r. spectra. *J Chem Soc Chem Commun*, 694-696.

Alberts, A. W., J. Chen, G. Kuron & other authors (1980). Mevinolin: A highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. *Proc Natl Acad Sci USA* **77**, 3957-3961.

Alspaugh, J. A., Perfect, J. R. & Heitman, J. (1997). Cryptococcus neoformans mating and virulence are regulated by the G-protein alpha subunit GPA1 and cAMP. *Genes Dev* **11**, 3206-3217.

Anderson, J. A. & Chung, C. H. (1990). Conversion of versiconal acetate to versicona[and versicolorin C in extracts from *Aspergillus parasiticus*. *Mycopathologia* **110**, 31-35.

Anderson, J. A. (1992). Enzymes in aflatoxin biosynthesis. *World Journal of Microbiology and Biotechnology* **8**, 96-98.

Anke, T. & Thines, E. (2007). *Fungal metabolites as lead structures for agriculture*: CUP.

Antonia, G., Giancarlo, P., Michele, S., Filomena, E., Abdelhamid, A., Alan, D. W. D. & Giuseppina, M. (2008). Characterisation of a pks gene which is expressed during ochratoxin A production by *Aspergillus carbonarius*. *Int J Food Microbiol* **129**, 8-15.

Atoui, A., Dao., P., Mathieu, F. & Lebrihi, A. (2006). Amplification and diversity analysis of Ketosynthase domains of putative polyketide synthase gene in *Aspergillus ochraceus* and *Aspergillus carbonarius* producers of ochratoxin A. *Mol Nutr Food Res* **50**, 448-493.

Auclair, K., Kennedy, J., Hutchinson, C. R. & Vederasa, J. C. (2001). Conversion of Cyclic Nonaketides to Lovastatin and Compactin by a lovC Deficient Mutant of *Aspergillus terreus*. **11**, 1527-1531.

Awad, G., Mathieu, F., Coppel, Y. & Lebrihi, A. (2005). Characterization and regulation of new secondary metabolites from *Aspergillus ochraceus* M18 obtained by UV mutagenesis. *Can J Microbiol* **51**, 59-67.

Axberg, K. & Catenbeck, S. (1975). Intermediates in the penicillic acid biosynthesis in *Penicillium cyclopium*. *Acta Chem Scand B* **29**, 749-751.

Axberg, K. & Gatenbeck, S. (1975). Intermediates in the penicillic acid biosynthesis in Penicillium cyclopium. *Acta Chem Scand Ser* **29**, 749-751.

Bacha, N., Atoui, A., Mathieu, F., Liboz, T. & Lebrihi, A. (2009a). Aspergillus westerdijkiae polyketide synthase gene "aoks1" is involved in the biosynthesis of ochratoxin A. *Fungal Genetics and Biology* **46**, 77-84.

Bacha, N., Dao, H. P., Atoui, A., Mathieu, F., O'Callaghan, J., Puel, O., Liboz, T., Dobson, A. D. W. & Lebrihi, A. (2009b). Cloning and characterization of novel methylsalicylic acid synthase gene involved in the biosynthesis of isoasperlactone and asperlactone in Aspergillus westerdijkiae. *Fungal Genet Biol* **46**, 742-749.

Baker, S. E., Kroken, S., Asvarak, T., Inderbitzin, P., Li, B. Y., Shi, L., Yoder, O. C. & Turgeon, B. G. (2006). Two polyketide synthase-encoding genes are required for biosynthesis of the polyketide virulence factor, T-toxin, by *Cochliobolus heterostrophus*. *Mol Plant Microbe Interact* **19**, 139-149.

Balcells, M., Canela, R., Coll, j., Sanchis, V. & Torres, M. (1995). Effect of fungal metabolites and some derivatives against *Tribolium castaneum* (Herbst) and *Nezara viridula* (L.). *Pestic sci* **45**, 319-323.

Balcells, M., Canela, R., Sala, N., Sanchis, V. & Torres, M. (1998). Bactericidal and fungicidal activity of *Aspergillus westerdijkiae* metabolites and some derivatives. *Pestic sci* **53**, 00009-00014.

Baldwin, J. E., Blackburn, J. M., Sutherland, J. D. & Wright, M. C. (1991). High-level soluble expression of isopenicillin-N synthase isozymes in E. coli. *Tetrahedron Letters* **47**, 5991-6002.

Bassett, E. W. & Tanenbaum, S. W. (1958). The biosynthesis of patulin. II. The general physiology of several strains of *Penicillium patulum*. *Biochim Biophys Acta* **28**, 247-260.

Bastin, R. & Roey, G. V. (1954). Action of some antibiotics and inhibiting substances on cellular stretching. *P Pharm Belg* **9**, 112-127.

Batista, L. R., Chalfoun, S. M., Prado, G., Schwan, R. F. & Wheals, A. E. (2003). Toxigenic fungi associated with processed (green) coffee beans (Coffea arabica L.). *Int J Food Microbiol* **85**, 293-300.

Battilani, P., Pietri, A., Bertuzzi, A., Languasco, L., Giorni, P. & Kozakiewicz, Z. (2003). Occurrence of ochratoxin A-producing fungi in grapes grown in Italy. *J Food Prot* **66**, 633-666.

Bayman, P., Baker, J. L., Doster, M. A., Michaillides, T. J. & Mahony, N. E. (2002). Ochratoxin production by the *Aspergillus ochraceus* group and *Aspergillus alliaceus*. *Appl Environ Microbiol* **68**, 2326-2329.

Beck, J., Ripka, S., Siegner, A., Schiltz, E. & Schweizer, E. (1990). The multifunctional 6-methylsalicylic acid synthase gene of *Penicillium patulum*. Its gene structure relative to that of other polyketide synthases. *Eur J Biochem* **192**, 487–498.

Bennett, J. W. & Papa, K. E. (1988). *Genetics of plant pathology*. London, United Kingdom: Academic Press.

Bentley, R. & Bennett, J. (1999). Constructing polyketides: From Collie to combinatorial biosynthesis. *Annu Rev Microbiol* **53**, 411-446.

Berdy, J. (1974). Are actinomycetes exhausted as a source of secondary metabolites? In *Proceedings of the 9th International Symposium on the biology of actinomycetes Part 1*, pp. 3-23. NewYork: Allerton Press.

Bhatnagar, D., Ullah, A. H. J. & Cleveland, T. E. (1988). Purification and characterization of a methyl transferase from *Aspergillus parasiticus* SRRC 163 involved in aflatoxin biosynthetic pathway. *Preparative Biochemistry* **18**, 321-349.

Bhatnagar, D., Cleveland, T. E. & Lillehoj, E. B. (1989). Enzymes in aflatoxin B1 biosynthesis: strategies for identifying pertinent genes. *Mycopathologia* **107**, 75-83.

Bhatnagar, D., Ehrlich, K. C. & Cleveland, T. E. (2003). Molecular genetic analysis and regulation of aflatoxin biosynthesis. *Appl Microbiol Biotechnol* **61**, 83-93.

Bingle, L. E., Simpson, T. J. & Lazarus, C. M. (1999). Ketosynthase domain probes identify two subclasses of fungal polyketide synthase genes. *Fungal Genet Biol* **26**, 209-223.

Birch, A. J. & Donovan, F. W. (1953). Studies in relation to Biosynthesis. I. Some possible routes to derivatives of Orcinol and Phloroglucinol. *Aust J Chem* **6**, 360-368.

Boutrif, E. & Canet, C. (1998). Mycotoxin prevention and control: FAO programmes. *Rev Méd Vét* **149** 681-694.

Brereton, R. G., Garson, M. J. & Staunton, J. (1984). Biosynthesis of fungal metabolites: asperlactone and its relationship to other metabolites of Aspergillus melleus.

Journal of the Chemical Society, Perkin Transactions 1: Organic and Bio-organic Chemistry **5**, 1027-1033.

Bridge, P. D., Arora, D. K., Reddy, C. A. & Elander, R. P. (1998). *Applications of PCR in mycology*: CAB International. UK.

Brodnik, T. (1975). Influence of toxic products of Fusarium graminearum and Fusarium moniliforme on maize seed germination and embryo growth. *Seed Sci Technol* **3**, 691-696.

Bruner, S. D., Weber, T., Kohli, R. M., Schwarzer, D., Marahiel, M. A., Walsh, C. T. & Stubbs, M. T. (2002). Structural Basis for the Cyclization of the Lipopeptide Antibiotic Surfactin by the Thioesterase Domain SrfTE. *structure* **10**, 301-310.

Bu'Lock, J. D., Sheperd, D. & Winstanley, D. J. (1968). Regulation of 6-methylsalicylate and patulin synthesis in Penicillium urticae. *Can J Microbiol* **15**, 279 - 285.

Bu'Lock, J. D. (1961). Intermediary metabolism and antibiotic synthesis. *Adv Appl Microbiol* **3**, 293-342.

Budavari, S. E. (1989). *The Merck Index*, 11 edn: Merck Co. Inc., Rahway, NJ.

Butler, M. J. & Day, A. W. (1998). Fungal melanins: a review. *Can J Microbiol* **44**, 1115-1136.

Calvo, A. M., Gardner, H. W. & Keller, N. P. (2001). Genetic connection between fatty acid metabolism and sporulation in Aspergillus nidulans. *J Biol Chem* **276**, 20766-20774.

Calvo, A. M., Wilson, R. A., Bok, J. W. & Keller, N. P. (2002). Relationship between Secondary Metabolism and Fungal Development. *Microbiol Mol Biol Rev* **66**, 447-459.

Carlile, M. J. & Watkinson, S. C. (1997). *The fungi*. San diego CA: Academic Press.

Cary, J. W., Ehrlich, K. C., Bland, J. M. & Montalbano, B. G. (2006). The Aflatoxin Biosynthesis Cluster Gene, aflX, Encodes an Oxidoreductase Involved in Conversion of Versicolorin A to Demethylsterigmatocystin. *App Environ Microbiol* **72**, 1096-1101.

Chadha, B. S. & Garcha, H. S. (1992). Mixed cultivation of Trichoderma reesei and Aspergillus ochraceus for improved cellulase production. *Acta Microbiol Hung* **39**, 61-67.

Champe, S. P., Rao, P. & Chang, A. (1987). n endogenous inducer of sexual development in Aspergillus nidulans. *A J Gen Microbiol* **133**, 1383-1388.

Champe, S. P. & El-Zayat, A. A. E. (1989). Isolation of a sexual sporulation hormone from Aspergillus nidulans. *J Bacteriol* **171**, 3982-3988.

Chen, K. C. Y., W S : Tiu, C : Houng, J Y (1994). 11 alpha-hydroxylation of progesterone using modified alginate-immobilized cells. *Enzyme MicrobTechnol* **16**, 551-555.

Christensen, M. & Tuthill, D. E. (1985). *Aspergillus: an overview In:*. Samson RA, Pitt JI, eds. Advances in Pencillium and Aspergillus systematics: NY: Plenum Press.

Chuturgoon, A. A., Dutton, M. F. & Berry, R. K. (1990). The preparation of an enzyme associated with aflatoxin biosynthesis by affinity chromatography. *Biochem and Biophys Res Com* **166**, 38-42.

Ciegler, A. & Kurtzman, C. P. (1970). Penicillic acid production by blue-eye fungi on various agricultural commodities. *Appi Microbiol* **20**, 761-764.

Ciegler, A., Mintzlaff, H. J., Weisleder, D. & Leistner, L. (1972). Potential Production and Detoxification of Penicillic Acid in Mold-Fermented Sausage (Salami). *Appl Microbiol* **24**, 114-119.

Clegler, A., Detroy, R. W. & LUllehoj, E. B. (1971). *Patulin, penicillic acid, and other carcinogenic lactones*. New York: Academic Press, Inc.

Cole, R. J., H, M. J., D, D. N., W, K. J. & L, D. U. (1971). 4-Hydroxymellein: a new metabolite of *Aspergillus ochraceus*. . *J Agr Food Chem Biol* **19**, 909-911.

Correia, T., Grammel, N., Ortel, I., Keller, U. & Tudzynski, P. (2003). Molecular cloning and analysis of the ergopeptine assembly system in the ergot fungus *Claviceps purpurea*. *Chem Biol* **10**, 1281-1292.

Cosmina, P., Rodriguez, F., de Ferra, F., Grandi, G., Perego, M., Venema, G. & van Sinderen, D. (1993). Sequence and analysis of the genetic locus responsible for surfactin synthesis in *Bacillus subtilis*. *Mol Microbiol* **8**, 821-831.

Cox, R. J. (2000). Biosynthesis. *Annu Rep Prog Chem, Sect B* **96**, 231-258.

Cullen, D., Leong, S. A., Wilson, L. J. & Henner, D. J. (1987). Transformation of *Aspergillus nidulans* with the hygromycin-resistance gene, hph. *Gene* **57**, 21-26.

Dalcero, A., Magnoli, C., Hallak, C., Chiacchiera, S. M., Palacio, G. & Rosa, C. A. (2002). Detection of ochratoxin A in animal feeds and capacity to produce this mycotoxin by *Aspergillus* section Nigri in Argentina. *Food Addit Contam* **19**, 1065-1072.

Dao, H. P., Mathieu, F. & A, L. (2005). Two primer pairs to detect OTA producers by PCR method. *Int J Food Microbiol* **104**, 61-67.

David, J. B., Eckhart, S., David, A. H. & Chaitan, K. (1995). Expression of a functional fungal polyketide synthase in the bacterium *Streptomyces coelicolor* A3(2). *J Bacteriol* **177**, 4544–4548.

Davis, N. D., Searcy, J. W. & L, D. U. (1969). Production of ochratoxin A by *Aspergillus ochraceus* in a semisynthetic medium. *Appl Microbiol* **17**, 742-744.

de Crecy-Lagard, V., Saurin, W., Thibaut, D., Gil, p., Naudin, l., Crouzet, J. & Blanc, V. (1997). Streptogramin B biosynthesis in *Streptomyces pristinaespiralis* and *Streptomyces virginiae*: molecular characterization of the last structural peptide synthetase gene. . *Antimicrob Agents Chemother* **41**, 1910-1915.

Dickens, F. & Jones, H. E. H. (1961). Carcinogenic activity of a series of reactive lactones and related substances. *Br J Cancer* **15**, 85-88.

Dinroth, P., Ringelmann, E. & Lynen, F. (1976). Methylsalicylic acid synthetase from Penicillium patulum. Some catalytic properties of the enzyme and its relation to fatty acid synthetase. *Eur J Biochem* **68**, 591-596.

Doull, J. L., Ayer, S. W., Singh, A. K. & Thibault, P. (1993). Production of a novel polyketide antibiotic, Jadomycin B, by *Streptomyces venezuelae* following heat shock. *J Antibiot* **46**, 869-871.

Du, L. & Shen, B. (2001). Biosynthesis of hybrid peptide-polyketide natural products. *Curr Opin Drug Discovery Dev* **4**, 215-228.

Eisendle, M., Oberegger, H., Zadra, I. & Haas, H. (2003). The siderophore system is essential for viability of *Aspergillus nidulans*: functional analysis of two genes encoding l-ornithine N 5-monooxygenase (sidA) and a non-ribosomal peptide synthetase (sidC). *Mol Microbiol* **49**, 359-375.

El-Dessouki, S. (1992). Ochratoxin A in bier. *Deutsche Lebensmittel-Rundschau* **11**, 354-355.

Eley, K. L., Halo, L. M., Song, Z., Powles, H., Cox, R. J., Bailey, A. M., Lazarus, C. M. & Simpson, T. J. (2007). Biosynthesis of the 2-Pyridone tenellin in the Insect pathogenic fungus *Beauveria bassiana*. *Chem Bio Chem* **8**, 289-297.

Ellen, M. F. & Barbara, J. H. (2008). Secondary metabolism: regulation and role in fungal biology. *Curr Opin Microbiol* **11**, 481-487.

FAO Food and Nutrition Paper No 81 (2004). Worldwide Regulations for Mycotoxins in Food and Feed in 2003, pp. 180 pages.

Färber, P. & Geisen, R. (2004). Analysis of Differentially-Expressed Ochratoxin A Biosynthesis Genes of Penicillium Nordicum. *Eur J Plant Pathol* **110**, 661-669.

Feng, G. H. & Leonard, T. J. (1998). Culture conditions control expression of the Genes for aflatoxin and sterigmatocystin biosynthesis in *Aspergillus parasiticus* and *A. nidulans*. *Appl Environ Microbiol* **64**, 2275-2277

Forrester, P. I. & Gaucher, G. M. (1972). Conversion of 6-methylsalicylic acid into patulin by Penicillium urticae. *Biochemistry* **11**, 1102-1107.

Frisvad, J. C., Frank, J. M., Houbraken, J. A. M. P., Kuijpers, A. F. A. & Samson, R. A. (2004a). New ochratoxin A producing species of *Aspergillus section Circumdati*. *Stud Mycol* **50**, 23–43.

Frisvad, J. C., Skouboe, P. & Samson, R. A. (2004b). A new efficient producer of aflatoxin B1, sterigmatocystin and 3-O-methylsterigmatocystin, *Aspergillus rambellii* sp. *nov Systemat Appl Microbiol* **28**, 442-453.

Fujii, I., Watanabe, A., Sankawa, U. & Ebizuka, Y. (2001). Identification of Claisen cyclase domain in fungal polyketide synthase WA, a naphthopyrone synthase of *Aspergillus nidulans*. *Chem Biol* **8**, 189-197.

Gaisser, S., Trefzer, A., Stockert, S., Kirschning, A. & Bechthold, A. (1997). Cloning of an avilamycin biosynthetic gene cluster from *Streptomyces viridochromogenes* Tü57. *J of Bacteriol* **179**, 6271-6278.

Gardes, M. & Bruns, T. D. (1993). TS primers with enhanced specificity for Basidiomycetes-applicatin to the identification of mycorrhizae and rusts. *Mol Ecol* **2**, 113-118.

Garson, M. J., Staunton, J. & Jones, P. G. (1984). New polyketide metabolites from Aspergillus melleus: structural and stereochemical studies. *Journal of the Chemical Society, Perkin Transactions 1: Organic and Bio-organic Chemistry (1972-1999)* **5**, 1021-1026.

Gaucher, G. M. & Shepherd, M. G. (1968). Isolation of orsellinic acid synthase. *Biochem and Biophys Res Com* **32**, 664-671.

Gaucher, G. M. (1979). Mycotoxins-their biosynthesis in fungi: patulin and related carcinogenic lactones. *J Food Prot* **42**, 810-814.

Geisen, R. (1996). Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. *System Appl Microbiol* **19**, 388-392.

Geisen, R., Mayer, Z., Karolewiez, A. & Farber, P. (2004). Development of a Real Time PCR System for detection of Penicillium nordicum and for Monitoring Ochratoxin A production in foods by targeting the ochratoxin polyketide synthase gene. *System Appl Microbiol* **27**, 501-504.

Geoffrey, T. (2000). Exploitation of fungal secondary metabolites old and new. *Microbiology today* **27**, 118-120.

Gokhale, R. S. & Tuteja, D. (2001). Biochemistry of polyketide synthases. *Biotechnology* **10**, 341-372.

Graziani, S., Vasnier, C. & Daboussi, M.-J. (2004). Novel Polyketide Synthase from *Nectria haematococca*. *Appl Environ Microbiol* **70**, 2984–2988.

Grootwassink, J. W. D. & Gaucher, G. M. (1980). De novo biosynthesis of secondary metabolism enzymes in homogeneous cultures of Penicillium urticae. *J Bacteriol* **141**, 443-455.

Guzman-de-Pen˜a, D., Aguirre, J. & Ruiz-Herrera, J. (1998). Correlation between the regulation of sterigmatocystin biosynthesis and asexual and sexual sporulation in Emericella nidulans. *Antonie Leeuwenhoek* **73**, 199-205.

Haese, A., M, Schubert, M., Herrmann & Zocher, R. (1993). Molecular characterization of the enniatin synthetase gene encoding a multifunctional enzyme catalysing N methyldepsipeptide formation in Fusarium scirpi. *Mol Microbiol* **7**, 905-914.

Harris, J. P. & Mantle, P. G. (2001). Biosynthesis of ochratoxins by *Aspergillus ochraceus*. *Phytochemistry* **58**, 709-716.

Hayes, A. W. (1980). Mycotoxins: A review of biological effects and their role in human diseases. *Clinical Toxicol* **17**, 45 - 83.

Hendrickson, L., Davis, C. R., Roach, C., Nguyen, D. K., Aldrich, T., McAda, P. C. & Reeves, C. D. (1999). Lovastatin biosynthesis in *Aspergillus terreus*: characterization of blocked mutants, enzyme activities and a multifunctional polyketide synthase gene. *Chem Biol* **6**, 429-439.

Herrmann, M., Zocher, R. & Haese, A. (1996). Effect of disruption of the enniatin synthetase gene on the virulence of Fusarium avenaceum. *Mol Plant-Microbe Interact* **9**, 226-232.

Hicks, J., J.-H, Yu, N. K. & Adams, T. H. (1997). Aspergillus sporulation and mycotoxin production both require inactivation of the FadA Galpha protein-dependent signaling pathway. *EMBO J* **16**, 4916-4923.

Hopwood, D. & Sherman, D. (1990). Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. *Annu Rev Genet* **24**, 37-66.

Hopwood, D. A. (1988). towards an understanding of gene switching in Streptomyces, the basis of sporulation and antibiotic production. *T Proc R Soc Lond* **235**, 121-138.

Hsieh, D. P. H., Wan, C. C. & Billington, J. A. (1989). A versiconal hemiacetal acetate converting enzyme in aflatoxin biosynthesis. *Mycopathology* **107**, 121-126.

Huff, E. E. & Hamilton, P. (1979). Mycotoxin-their biosynthesis in fungi: ochratoxin metabolites of combined pathways. *J Food Port* **42**, 815-820.

IARC (1993). Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines, and Mycotoxins. In *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans*, pp. 571.

Isao, F., Akira, W., Yuichiro, M. & Yutaka, E. (1998). Structures and Functional Analyses of Fungal Polyketide Synthase Genes. *Actinomycetol* **12**, 1-14.

J. Kennedy, K. Auclair, S. G. Kendrew, C. Park, J.C. Vederas & Hutchinson, C. R. (1999). Modulation of polyketide synthase activity by accessory proteins during lovastatin biosynthesis. *Science* **284**, 1368-1372.

Jacqueline, K. & Daniel, T. (1985). Role of Penicillic Acid in the Phytotoxicity of *Penicillium cyclopium* and *Penicillium canescens* to the Germination of Corn Seeds. **49**, 660-663.

James, R. H. (2008). *The Chemistry of Fungi*: Royal Society of Chemistry.

James, S. & Andrew, C. S. (1991). 17O NMR in biosynthetic studies: aspyrone, asperlactone and isoasperlactone, metabolites of *Aspergillus melleus*. *J Chem Soc, Chem Commun*, 1106-1108.

Jaoanne, W. S. H., Corentin, H., Nafees, B., Richard, O. C., Claude, L., Giséle, B., Michel, R., Pierre, R. & Bernard, D. (2007). Regulation and role of the STE12-like transcription factor from the plant pathogen *Colletotrichum lindemuthianum*. *Mol Microbiol* **64**, 68-82.

Jin, H. D. & Dong-K, C. (2007). Aflatoxins: Detection, Toxicity, and Biosynthesis. *Biotechnol & bioproc eng* **12**, 585-593.

Jing, Z., Ning, Y. & Runying, Z. (2008). Phylogenetic analysis of type I polyketide synthase and nonribosomal peptide synthetase genes in *Antarctic sediment*. *Extremophiles* **12**, 97-105.

Jiujiang, Y., Perng-Kuang, C., Kenneth, C. E. & other authors (2004). Clustered Pathway Genes in Aflatoxin Biosynthesis. *App Environ Microbiol* **70**, 1253-1262.

John, L. S., Karine, A., Jonathan, K., C. Richard, H. & John, C. V. (2002). Transformations of cyclic nonaketides by Aspergillus terreus mutants blocked for lovastatin biosynthesis at the lovA and lovC genes. *Org Biomol Chm* **1**, 50-59.

Johnson, R. D., Johnson, L., Itoh, Y., Kodama, M., Otani, H. & Kohmoto, K. (2000). Cloning and characterization of a cyclic peptide synthetase gene from *Alternaria alternata* apple pathotype whose product is involved in AM-toxin synthesis and pathogenicity. *Mol Plant-Microbe Interact* **13**, 742-753.

Jorgensen, K. (1998). A survey of pork, poultry, coffee, beer and pulses for Ochratoxin A. *Food Addit Contam* **15**, 550-554.

Jorgensen, K. & Jacobsen, J. S. (2002). Occurrence of ochratoxin A in Danish wheat and rye, 1992-1999. *Food Addit Contam* **19**, 1184-1189.

Julia, S. & christian, H. (2007). Molecular basis of Cytochalasan biosynthesis in fungi: Gene cluster analysis and evidence for the involvement of a PKS-NRPS hybrid synthase by RNA silencing. *American chemical society* **129**, 9564-9565.

Kang, S. W. & Kim, S. W. (2004). New antifungal activity of pinicillic acid against phytophthora species. *Biotechnol Lett* **26**, 695-698.

Kao, C. M., Katz, L. & Khosla, C. (1994). Engineered biosynthesis of a complete macrolactone in a heterologous host. *Science* **265**, 509-512.

Karolewiez, A. & Geisen, R. (2005). Cloning a part of the ochratoxin A biosynthetic gene cluster of Penicillium nordicum and characterisation of the ochratoxin polyketide synthase gene. *Syst Appl Microbiol* **28**, 588-595.

Kawaguchi, T., Azuma, M., Horinouchi, S. & Beppu, T. (1988). Effect of B-factor and its analogues on rifamycin biosynthesis in *Nocardia* sp. *J Antibiot* **41**, 360-365.

Kawamura, C., Tsujimoto, T. & Tsuge, T. (1999). Targeted disruption of a melanin biosynthesis gene affects conidial development and UV tolerance in the Japanese pear pathotype of Alternaria alternata. *Mol Plant-Microbe Interact* **12**, 59-63.

Keller, N. P. & Hohn, T. M. (1997). Metabolic pathway gene clusters in filamentous fungi. *Fungal Genet Biol* **21**, 17-29.

Kennedy, J., Auclair, K., Kendrew, S. G., Park, C., Vederas, J. C. & Hutchinson, C. R. (1999). Modulation of polyketide synthase activity by accessory proteins during lovastatin biosynthesis. *Science* **284**, 1368-1372.

Khosla, C., Gokhale, R. S., Jacobsen, J. R. & Cane, D. E. (1999). Tolerance and specificity of polyketide synthases. *Annu ReV Biochem* **68**, 219-253.

Kiffer, E. & Morelet, M. (1997). Les deutéromycètes: Institut National de la Recherche Agronomique.

Kim, Y. T., Lee, Y. R., Jin, J., Han, K. H., Kim, H., Kim, J. C., Lee, T., Yun, S. H. & Lee, Y. W. (2005). Two different polyketide synthase genes are required for synthesis of zearalenone in *Gibberella zea*. *Mol Microbiol* **58**, 1102-1113.

Kleinkauf, H. & Doehren, H. V. (1996). A nonribosomal system of peptide biosynthesis. *Eur J Biochem* **236**, 335-351.

Korzybski, T., Kowszyk-Gindifer, Z. & Kuryowicz, W. (1978). *Antibiotics: origin, nature, and properties,*. Washington, D.C: American Society for Microbiology.

Kozakiewicz, Z. (1989). Aspergillus species on stored products. *Mycol Pap* **161**, 1-188.

Krogh, P. (1987). *Ochratoxin A in food. Mycotoxin in Food*. San Diego: Academic Press.

Kroken, S., Glass, N. L., Taylor, J. W., Yoder, O. C. & Turgeon, B. G. (2003). Phylogenomic analysis of type I polyketide synthase genes in pathogenic and saprobic ascomycetes. *PNAS* **100**, 15670-15675.

Kuiper-Goodman, T. & Scott, P. M. (1989). Review: risk assessment of the mycotoxin ochratoxin A. *Biomed Environ Sci* **2**, 179-248.

Kurtzman, C. P. & Ciegler, A. (1970). Mycotoxin from a blue-eye mold of corn. *Appl Microbiol* **20**, 204-207.

Kuwayama, H., Shinji, O., Takahiro, M., Mariko, K., Hideko, U. & Yoshimasa, T. (2002). PCR-mediated generation of a gene disruption construct without the use of DNA ligase and plasmid vectors. *Nucleic Acids Res* **30**, 2e2.

Le Bars, J. (1979). Contamination alimentaire par le *Penicillium cyclopium* Westling. Frequence de souches productrices d'acide penicillique. *Ann Rech Vet* **10**, 119-124.

Le Bars, J. (1980). Facteurs favorisant la production d'acide penicillique par *Penicillium verrucosum* var. cyclopium Westling dans les denrees alimentaires. *Ann Rech Vet* **11**, 321-326.

Le Bars, J. (1982). Facteurs de l'accumulation d'acide penicillique dans les denrdes d'origine vegetale. *Sci Aliment* **2**, 29-33.

Lee, T., Oh, D. W., Kim, H. S., Lee, J., Kim, Y. H., Yun, S. H. & Lee, Y. W. (2001a). Identification of deoxynivalenol- and nivalenol-producing chemotypes of *Gibberella zeae* by using PCR *Appl Environ Microbiol* **67**, 2966-2972.

Lee, T., Yun, S. H., Hodge, K. T., Humber, R. A., Krasnoff, S. B., Turgeon, G. B., Yoder, O. C. & Gibson, D. M. (2001b). Polyketide synthase genes in insect- and nematode-associated fungi. *Appl Microbiol Biotechnol* **56**, 181-187.

Liou, G. F. & Khosla, C. (2003). Building-block selectivity of polyketide synthases. *Curr Opin Chem Biol* **7**, 279-284.

Lui, D., Caloe, C., Biard, R. & Pedersen, J. (2000). Rapid mini preparation of fungal DNA for PCR. *J Clin Microboil* **38**, 471.

Machida, M., Asai, K., Sano, M. & Tanaka, T. (2005). Genome sequencing and analysis of Aspergillus oryzae. *Nature* **438**, 1157-1161.

Madhyastha, M. S., Marquardt, R. R., Masi, A., Borsa, J. & Frohlich, A. A. (1994). Comparison of toxicity of different mycotoxins to several species of bacteria and yeasts : use of *Bacillus brevis* in a disc diffusion assay. *J Food Port* **57**, 48-53.

Magan, N. & Olsen, M. (2004). *Mycotoxins in Food: Detection and Control*: Cambridge: DC/Woodhead Publishing Limited.

Majerus, P., Cutko, I., Dreyer, A., El-Dessouki, S., Eyrich, W., Reusch, H., Schurer, B. & Waiblinger, H. U. (1993). Zur Belastungssituation von Ochratoxin A, in Lebensmitteln pflanzlichen Ursprungs. *Deutesche Lebensmitteln-Rundschau* **89**, 112-113.

Mapleston, R. A., Stone, M. J. & Williams, D. H. (1992). The evolutionary role of secondary metabolites. *Gene* **115**, 151-157.

Marchand, C., Johnson, A., Karki, R. G., Pais, G. C. G., Zhang, X. & Cowansage, K. (2003). Metal-dependent inhibition of HIV-1 integrase by β-diketo acids and resistance of the soluble double-mutant (F185K/C280S). *Mol Pharmacol* **64**, 600-609.

Mary, J., Garson, Staunton, J. & Peter, G. J. (1984). New polyketide metabolites from *Aspergillus melleus*: structural and stereochemical studies. *J Chem Soc Perkin Trans* **1**, 1021 – 1026.

Mathias, M. & Dirk, H. (2007). Fungal Genetics, Genomics, and Secondary Metabolites in Pharmaceutical Sciences. *planta Med* **73**, 103-115.

Mathivanan, N., Prabavathy, V. R. & Vijayanandraj, V. R. (2008). *The Effect of Fungal Secondary Metabolites on Bacterial and Fungal Pathogens*. Berlin Heidelberg: Springer

Mazur, P., Nakanishi, K., El-Zayat, A. A. E. & Champe, S. P. (1991). Structure and synthesis of sporogenic psi factors from Aspergillus nidulans. *J Chem Soc Chem Commun* **20**, 1486-1487.

McDaniel, R., Ebert-Khosla, S., Hopwood, D. A. & Khosla, C. (1993). Engineered biosynthesis of novel polyketides. *Science* **262**, 1546-1550.

McQuire, S. M., BrobsL, S. W., Graybilt, T. L., Pal, K. & Townsend, C. A. (1989). Partitioning of tetrahydro- and dihydrobisfuran formation in aflatoxin biosynthesis defined by cell-free and direct incorporation studies. *Journal of the American Chemistry Society* **111**, 8308-8309.

Metz, J. G., Roessler, P., Facciotti, D. & other authors (2001). Production of polyunsaturated fatty acids by polyketide synthase in both prokaryotes and eukaryotes. *Science* **293**, 290-293.

Michito, T., Yoichi, I. & Hisae, M. (1996). Herbicide. *Japan Patent*, 08-053310.

Moffitt, M. C., Neilan, B. A. & (2003). Evolutionary affiliations within the superfamily of ketosynthases reflect complex pathway associations. *J Mol Evol* **56**, 446-457.

Moor, J. H., Davis, N. D. & Diener, U. L. (1972). Mellein and 4-Hydroxymellein Production by *Aspergillus ochraceus* Wilhelm1
app morobiol **23**, 1067-1072.

Mosbach, K. (1969). Zur Biosynthese roy Flechtenstoffen, Produkten einer symbiotischen Lebensgemeinschaft. *Angew Chem* **81**, 233-244.

Nancy, P. K., Robert, A. E., Butchko, Bashir, S. & Timothy, D. P. (1994). A Visual Pattern of Mycotoxin Production in Maize Kernels by *Aspergillus* spp. *Phytopathology* **84**, 483-488.

Nasser, Y., Daniel, J., Altier, E., Barbour, A., Cigan, L. & Christopher, J. S. (2001). Production of 6-Methylsalicylic Acid by Expression of a Fungal Polyketide Synthase Activates Disease Resistance in Tobacco. *plant cell* **13**, 1401-1410.

Nicholson, T. P., Rudd, B. A. M., Dawson, M., Lazarus, C. M., Simpson, T. J. & Cox, R. J. (2001). Design and utility of oligonucleotide gene probes for fungal polyketide synthases. *Chem Biol* **8**, 157-178.

Niermanm, W. C., Pain, A., Anderson, M. J. & other authors (2005). Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature* **438**, 1151-1156.

Nyfelder, R. & Keller, S. W. (1974). Metabolites of microorganisms, 143: echinocandin B, a novel polypeptide-antibiotic from Aspergillus nidulans var. echinulatus-isolation and structural components. *Helv Chim Acta* **57**, 2459-2477.

O'Callaghan, J., Caddick, M. X. & Dobson, A. D. W. (2003). A polyketide synthase gene required for ochratoxin A biosynthesis in *Aspergillus ochraceus*. *Microbiol* **149**, 3485-3491.

Ominski, K. H., Marquardt, R. R., Sinah, R. N. & Abramson, D. (1994). *Ecological aspects of growth and mycotoxin production by storage fungi*. St. Paul: Eagan Press.

Panagiotou, G., Andersen, M. R., Grotkjaer, T., Regueira, T. B., Nielsen, J. & Olsson, L. (2009). Studies of the production of fungal polyketides in Aspergillus nidulans by using systems biology tools. *Appl Environ Microbiol* **75**, 2212-2220.

Pel, H., J, W., Johannes, H. & other authors (2007). Genome sequencing and analysis of the versatile cell factory Aspergillus niger CBS 513.88. *Nature Biotechnol Pel* **25**, 221-231.

Perry, J. J., Staley, J. T. & Lory, S. (2004). *Microbiologie*. Dunod France.

Petzinger, E. & Ziegler, K. (2000). Ochratoxin A from a toxicological perspective. *J Vet Pharmacol Ther* **23**, 91-98.

Pohland, A. E., Nesheim, S. & Friedman, L. (1992). Ochratoxin A, a review. *Pure Appl Chem Biol* **64**, 1029-1046.

Puel, O., S.Tadrist, Delafoege, M., Oswald, I. P. & Lebrihi, A. (2007). The inability of *Byssochlamys fulva* to produce patulin is related to absence of 6-methylsalicylic acid synthase and isoepoxydon dehydrogenase genes. *Int J Food Microbiol* **115**, 131-139.

Puel, O. (2007a). Bases moleculare de la voie de biosyntheses de patuline, mycotoxine produit par Byssochlamys fulva et penicillium griseofulvum, pp. 230. Toulouse: Université de Toulouse 3.

Raper, K. B. & Fennell, D. I. (1965). *The genus Aspergillus.* Baltimore: Williams and Wilkins.

Reboux, G. (2006). Mycotoxins: health effects and relationship to other organic compounds. *Revue française d'allergologie et d'immunologie clinique* **46**, 208-212.

Reiss, J. (1979). Inhibitory action of the mycotoxins patulin and penicillic acid on urease. *Food Cosmet Tocicol* **17**, 145-146.

Ribelin, W. E., Fukushima, K. & Still, P. (1978). The toxicity of ochratoxin A to ruminants. *Canadian J Comp Med* **42**, 172-176.

Richard, J. P., Robert, B. B., Gary, S. L. & John, P. R. (1983). Microbial transformations of natural antitumor agents XXII: Conversion of bouvardin to O-desmethylbouvardin and bouvardin catechol. *J Pharm Sci* **72**, 1291-1293.

Robbers, J. E., Hong, S., Tuite, J. & Carlton, W. W. (1978). Production of xanthomegnin and viomellein by species of Aspergillus correlated with mycotoxicosis produced in mice. *App Environ Microbiol* **36**, 819-823.

Robert, H. P., Daren, W. B., Ronald, D. P. & Desjardins, A. E. (2003). Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in Gibberella moniliformis. *Fungal Genet Biol* **38**, 237-249.

Rosenbrook, W. J. & Carney, R. E. (1970). New metabolite from an unidentified *Aspergillus species*. *Tetrahedron Letters* **22**, 1867-1870.

Rossman, A. Y. (2009). The impact of invasive fungi on agricultural ecosystems in the United States. *Biol Invasions* **11**, 97-107.

Sambrook, J., Fritsch, E. F. & Maniatis , T. (1989). Molecular cloning: a laboratory Manual. Cold Spring Harbor: Cold Spring Harbor laboratory press.

Sasaki, M., Kaneko, Y., Oshita, K., Takamatsu, H., Asao, Y. & Yokotsuka, T. (1970). Studies on the compounds produced by molds. Part VII. Isolation of isocoumarin compounds. *Agr Biol Chem* **34**, 1296-1300.

Sassa, T., S. Hayakari, M. Ikeda & Y. Miura (1971). Plant growth inhibitors produced by fungi. I. Isolation and identification of penicillic acid and dihydropenicillic acid. *Agric Biol Chem Bio Chem* **35**, 2130-2131.

Schmidt-Heydt, M., Richter, W., Michulec, M., Buttinger, G. & Geisen, R. (2008). Comprehensive molecular system to study the presence, growth and ochratoxin A biosynthesis of Penicillium verrucosum in wheat. *Food Addit Contam* **25**, 989-996.

Schümann, J. & Hertweck, C. (2006). Advances in Cloning, Functional Analysis and Heterologous Expression of Fungal Polyketide Synthase Genes. *J Biotechnol*, in press.

Schwarzer, D. & Marahiel, M. A. (2001). Multimodular biocatalysts for natural product assembly. *Naturwissenschaften* **88**.

Schwecke, T., Aparicio, J. F., Molnár, I. & other authors (1995). The biosynthetic gene cluster for the polyketide immunosuppressant rapamycin. *PNAS* **92**, 7839-7843.

Scott-Craig, J. S., Panaccione, D. G., Pocard, J. A. & Walton, J. D. (1992). The cyclic peptide synthetase catalyzing HC-toxin production in the filamentous fungus Cochliobolus carbonum is encoded by a 15.7-kilobase open reading frame. *J Biol Chem* **267**, 26044-26049.

Searcy, J. W., Davis, N. D. & Diener, U. L. (1969). Biosynthesis of Ochratoxin A. *App Microbiol* **18**, 622-627.

Sebastian, B., Julia, S., Kirstin, S., Corinna, L., Axel, A. B. & Christian, H. (2007). Genomics-driven discovery of PKS-NRPS hybrid metabolites from *Aspergillus nidulans*. *Nature chem biol* **3**, 213-217.

Sekigouchi, J., Shimamot, T., Yamada, Y. & Gaucher, G. M. (1983). Patulin Biosynthesis: Enzymatic and Nonenzymatic Transformations of the Mycotoxin (E)-Ascladiol. *App Environ Microbiol* **45**, 1939-1942.

Sekiguchi, J. & Gaucher, G. M. (1977). Conidiogenesis and secondary metabolism in Penicillium urticae. *Appl Environ Microbiol* **33**, 147-158.

Sekiguchi, J., Katayama, S. & Yamada, Y. (1987). 6-Methyl-1,2,4-Benzenetriol, a new intermediate in Penicillic acid biosynthesis in *Penicillum cyclopium*. *App Environ Microbiol* **53**, 1531-1535.

Seo, J.-A., Proctor, R. H. & Plattner, R. D. (2001). Characterization of four clustered and coregulated genes associated with fumonisin biosynthesis in *Fusarium verticillioides*. *Fungal Genet Biol* **34**, 155-165.

Seshime, Y., Juvvadi, P. R., Fujii, I. & Kitamoto, K. (2005). Discovery of a superfamily of
type III polyketide synthase in Aspergillus oryzae. *Biochem Biophys Res Commun* **331**, 253-260.

Sidhu, G. S. (2002). Mycotoxin genetics and gene clusters. *Eur J of Plant Pathol* **108**, 705-711.

Sims, J. W., Fillmore, P., Warner, D. D. & Schmidt, E. W. (2005). Equisetin biosynthesis in *Fusarium heterosporum*. *Chem Commun*, 186-188.

Smith, D. J., M. K. Burnham, J. H. Bull & other authors (1990). Beta-lactam antibiotic biosynthetic genes have been conserved in clusters in prokaryotes and eukaryotes. *EMBO J* **9**, 741-747.

Smith, J. E. & Moss, M. (1985). *Mycotoxins: formation, analysis and significance*. United Kingdom: ohn Wiley & Sons.

Song, Z., Cox, R. J., Lazarus, C. & Simpson, M. T. J. (2004). Biosynthesis of Fusarin C in *Fusarium moniliforme* and *Fusarium venenatum*. *Chem Bio Chem* **5**, 1196-1203.

Sorensen, W. G. & Simpson, J. (1986). Toxicity of penicillic acid for rat alveolar macrophages in vitro. *Environ Res* **41**, 505-513.

Sorenson, W. G. & Simpson, J. (1986). Toxicity of penicillic acid for rat alveolar macrophages in vitro. *Environ Res* **41**, 505-513.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**, 503-517.

Stack, M. E. & Mislivec, P. B. (1978). Production of xanthomegnin and viomellein by isolates of Aspergillus ochraceus, Penicillium cyclopium, and Penicillium viridicatum. *App Environ Microbiol* **36**, 552-554.

Staunton, J. & Sutkowski, A. (1991). Oxygen-17 NMR in biosynthetic studies: aspyrone, asperlactone and isoasperlactone, metabolites of Aspergillus melleus. *J Chem Soc, Chem Commun* **16**, 1106-1108.

Steve, K. & Gary, P. (1999). Mycotoxins in Corn. North Carolina: College of agriculture and life sciences.

Steyn, P. S. & Holzapfel, C. W. (1970). Biosynthesis of ochratoxins, metabolites of Aspergillus ochraceus. *Phytochemistry* **9**, 1977-1983.

Stone, M. J. & Williams, D. H. (1992). On the evolution of functional secondary metabolites (natural products). *Mol Microbiol* **6**, 29-34.

Strohl, W. R. (1997). Industrial antibiotics: today and the future. In *Bio/technology of antibiotics*, pp. 1-47. New York: Marcel Dekkr.

Sua´rez-Quiroz, M., Gonza´les-Rios, O., Barel, M. & et al (2004). Study of ochratoxin A-producing strains in coffee processing. *Int J Food Sci Technol* **39**, 501-507.

Sun, C.-M. & Toia, R. F. (1993). Biosynthetic Studies on Ant Metabolites of Mellein and 2,4-Dihydroxyacetophenone from {1,2-13C2} Acetate. *J Nat Prod* **56**, 953-956.

Takano, Y., Kikuchi, T., Kubo, Y., Hamer, J. E., Mise, K. & Furusawa, I. (2000). The Colletotrichum lagenarium MAP kinase gene CMKI regulates diverse aspects of fungal pathogenesis. *Mol Plant-Microbe Interact* **13**, 374-383.

Tanenbaum, S. W. & Bassett, E. W. (1958). The biosynthesis of patulin. I. Related aromatic substances from Penicillium patulum, strain 2159A. *Biochim Biophys Acta* **28**, 21-31.

Tang, C. M., Cohen, J. & Holden, D. W. (1992). An *Aspergillus fumigatus* alkaline protease mutant constructed by gene disruption is deficient in extracellular elastase activity. *Molecular Microbiol* **6**, 1663-1671.

Tashiro, F., K. Hirai & Y. Ueno (1979). Inhibitory effects of carcinogenic mycotoxins on deoxyribonucleic acid-dependent ribonucleic acid polymerase and ribonuclease H. *App Environ Microbiol* **38**, 191-196.

Teren, J., Varga, J., Hamari, Z., Rinyu, E. & Kevei, F. (1996). Immunochemical detection of ochratoxin A in black Aspergillus strains. *Mycopathologia* **134**, 171-176.

Tintinalli, J. E., Kelen, G. D. & Stapczynski, J. S. (2004). Emergency Medicine: A Comprehensive Study Guide. New York: McGraw Hill.

Torres, M., Balcells, M., Sala, N., Sanchis, V. & Canela, R. (1998). Bactericidal and fungicidal activity of *Aspergillus ochraceus* metabolites and some derivatives. *Pesticide Science* **53**, 9-14.

Trail, F., Mahanti, N. & Linz, J. (1995). Molecular biology of aflatoxin biosynthesis. *Microbiology* **141**, 755-765.

Tudzynski, P., Holter, K., Correia, T., Arntz, C., Grammel, N. & Keller, U. (1999). Evidence for an ergot alkaloid gene cluster in *Claviceps purpurea*. *Mol Gen Genet* **261**, 133-141.

Umeda, M., Tsutsui, T. & Saito, M. (1977). Mutagenicity and inducibility of DNA single-strand breaks and chromosome aberrations by various mycotoxins. *Gann* **68**, 619-625.

Van der Merwe, K. J., Steyn, P. S. & Fourie, L. (1965). Mycotoxins Part II The constitution of ochratoxin A, B, and C, metabolites of Aspergillus ochraceus. *Wilh J Chem Soc*, 7083-7088.

van Loon, L. C., Bakker, P. A. H. M. & Pieterse, C. M. J. (1998). Systemic resistance induced by Rhizosphere bacteria. *Annu Rev Phytopathol* **36**, 453-483.

Varga, J., Kevei, E., Rimyu, E., Teren, J. & Kazakiewicz, Z. (1996). Ochratoxin production by Aspergillus species. *Appl Environ Microbiol* **62**, 4461-4464.

Varga, J., Rigo, K., Kocsube, S., Farkas, B. & Pal, K. (2003). Diversity of polyketide synthase gene sequences in *Aspergillus* species. *Res Microbiol* **154**, 593-600.

Vladimir, S., Paul, S. & Ilya, R. (1997). Airborne signalling by methyl salicylate in plant pathogen resistance. *Nature* **385**, 718-721.

von Dohren, H., Keller, U., Vater, J. & Zocher, R. (1997). Multifunctional peptide synthetases. *Chem Rev* **97**, 2675-2705.

W. F. Hoffman, A. W. Alberts, P. S. Anderson, J. S. Chen, R. L. Smith & A. K. Willard (1986). 3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors. 4. Side-chain ester derivatives of mevinolin. *J Med Chem* **29**, 849-852.

Wainwright, M. (1995). *An Introduction to Fungal Biotechnology*. Chichester: Wiley.

Walsh, C. T., Gehring, A. M., Weinreb, P. H., Quadri, L. E. & Flugel, R. S. (1997). Post-translational modification of polyketide and nonribosomal peptide synthases. . *Curr Opin Chem Biol* **1**, 309-315.

Webb, R. S. (1991). Process for the production of mellein and 4-hydroxymellein. United States.

Weber, G., Schorgendorfer, K., Schneider-Scherzer, E. & Leitner, E. (1994). the peptide synthetase catalyzing cyclosporine production in Tolypocladium niveum is encoded by a giant 45.8-kilobase open reading frame. *T Curr Genet* **26**, 120-125.

Weitnauer, G., Mühlenweg, A., Trefzer, A. & other authors (2001). Biosynthesis of the orthosomycin antibiotic avilamycin A: deductions from the molecular analysis of the avi biosynthetic gene cluster of *Streptomyces viridochromogenes* Tü57 and production of new antibiotics. *Chem Biol* **8**, 569-581.

Wiest, A., Grzegorski, D., Xu, B. W., Goulard, C., Rebuffat, S., Ebbole, D. J., Bodo, B. & Kenerley, C. (2002). Identification of peptaibols from *Trichoderma virens* and cloning of a peptaibol synthetase. *J Biol Chem* **277**, 20862-20868.

Yabe, K., Ando, Y. & Hamasaki, T. (1991). Desaturase activity in the branching step between aflatoxins B1 and G~ and aflatoxins B 2 and G. *Agricultural and Biological Chemistry* **55**, 1907-1911.

Yu, F., Zhu, X. & Du, L. (2005). Developing a genetic system for functional manipulations of FUM1, a polyketide synthase gene for the biosynthesis of fumonisins in *Fusarium verticillioides*. *FEMS Microbiol Lett* **248**, 257-264.

YU, J., CHANG, P.-K., CARY, J. W., WRIGHT, M., BHATNAGAR, D., CLEVELAND, T. E., PAYNE, G. A. & LINZ, J., E (1995). Comparative Mapping of Aflatoxin Pathway Gene Clusters in *Aspergillus parasiticus* and *Aspergillus flavus*. *App Environ Microbiol* **61**, 2365-2371.

Yu, J., Bhatnagar, D. & Cleveland, T. E. (2004a). Completed sequence of aflatoxin pathway gene cluster in Aspergillus parasiticus. *FEBS Letters* **564**, 126-130.

Yu, J. & Keller, N. (2005). Regulation of secondary metabolism in filamentous fungi. *Annu Rev Phytopathol* **43**, 437-458.

Yu, J. H. & Leonard, T. J. (1995). Sterigmatocystin biosynthesis in *Aspergillus nidulans* requires a novel type I polyketide synthase. *J Bacteriol* **177**, 4792-4800.

Yu, J. H., Hamari, Z., Han, K. H., Seo, J. A., Reyes-Domínguez, Y. & Scazzocchio, C. (2004b). Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. *Fungal Genetics and Biology* **41**, 973-981

Yuan, W. M., Gentil, G. D., Budde, A. D. & Leong, S. A. (2001). Characterization of the Ustilago maydis sid2 gene, encoding a multidomain peptide synthetase in the ferrichrome biosynthetic gene cluster. *J Bacteriol* **183**, 4040-4051.

Zamir, L. (1980). *he biosynthesis of patulin and penicillic acid*. New York: Academic Press, Inc.

Zhu, Peng, Yanling, Z., Yurong, Y., Xiaojun, Y. & Jianzhong, S. (2009). Molecular Phylogeny and Modular Structure of Hybrid NRPS/PKS Gene Fragment of

Pseudoalteromonas sp. NJ6-3-2 Isolated From Marine Sponge *Hymeniacidon perleve*. *J Mol Biol* **19**, 229-237.

Zimmerli, B. & Dick, R. (1996). Ochratoxin A in table wine and grape-juice: Occurrence and risk assessment. *Food Addit Contam* **13**, 655-668.

Characterization of the second polyketide synthase gene involved in the biosynthesis of ochratoxin A in *Aspergillus ochraceus* NRRL 3174

Nafees Bacha, Ali Atoui, Florence Mathieu and Ahmed Lebrihi
Département Bioprocédés et Systèmes Microbiens. Laboratoire de Génie Chimique, UMR 5503 (CNRS/INPT/UPS).
ENSAT-INP de Toulouse, 1, Avenue de l'Agrobiopôle, B.P. 32607, 31326 Castanet-Tolosan Cedex 1, France
E-mail address: lebrihi@ensat.fr

Introductio

Aspergillus ochraceus is a producer of various secondary metabolites including ochratoxin A (OTA) (Fig.1a), penicillic acid, viomellein, xanthomegnins and others polyketides such as mellein (Fig.1b) and spore pigments. Ochratoxin A is a main food contaminant and is possibly carcinogenic to humans (IARC (eds) (1993).
The biosynthetic pathway of OTA is not known to date. According to Huff and Hamilton (1979) mellein can be the precursor of OTA, but according to Harris and Mantle (2001) this hypothesis is not checked . In this study we described and characterized a PKS gene "*AoKS1*" (Accession Number: AY583209) in *Aspergillus ochraceus* NRRL 3174.

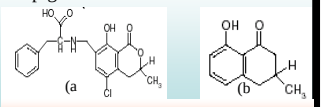


Figure 1: (a) Structure of OTA (b) and

Experimental techniques and Results

Description and characterization of AoKS1 gene

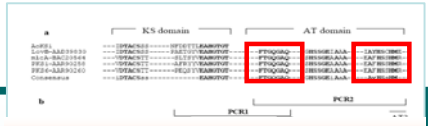
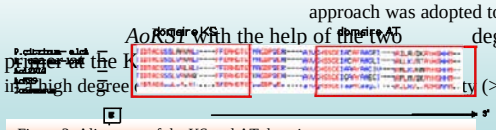


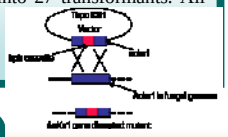
Figure 2: (a)Alignment of the KS domain with different sequences identified . (b) PCR based gene walking approach.

KS domain of *AoKS1* in *A. ochraceus* was identified with the help of a pair of primers KS1/KS2 designed for fungal and bacterial PKS. Alignment of the amino acid sequences of lovB, mlcA, pks1 and pks6 revealed two conserved regions in the AT domain, FTGQGAQ and AYHSSHM (Fig. 2a). These conserved regions were used to design two degenerated primers. Gene walking approach was adopted to sequence the 2 kb KS and AT domain of *AoKS1* with the help of the two degenerated AT primers and a specific primer at the KS domain. Alignment of the 2 kb sequenced AoKs1 resulted in high degree of identity (>50%) at the AT and KS domains of to lovB, mlcA and pks6.


Figure 3: Alignment of the KS and AT domains.

Disruption of AoKS1 gene and its

To determine the function of *AoKS1* gene, protoplasts of *A. ochraceus* were transformed with TopoKS1 vector containing hygromycine resistant cassette *(hph*) (Fig.4.) This disruption resulted into 27 transformants. All the transformants were screened by using PCR approach which resulted in 3 transformants disrupted in the AoKS1 region.


Figure 4: Disruption of *AoKS1*

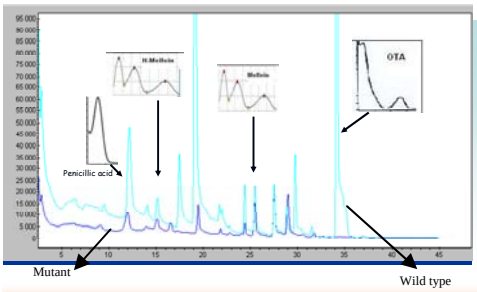


Figure 5: HPLC profile of wild type and AoKS1 mutant

Study of secondary metabolic

The HPLC (with fluorescence and UV detectors) profile of the 3 transformants in comparison to wild type showed that disruption of AoKS1 abolished the OTA production (Fig 5). All the 3 transformants showed the same profile as wild type for the production of mellein, 4-hydroxymellein (H-mellein) and the others secondary metabolites as penicillic acid.

Conclusio

O'Callaghan *et al.,* (2003) have previously cloned part of the polyketide synthase gene "*A.ochraceus* pks" required for OTA biosynthesis in *A. ochraceus* which is totally different from *AoKS1* (Fig.6.), but no information was provided by the authors concerning the presence or absence of some metabolites like the mellein. Based on this, we concluded that two pks may be involved in the biosynthesis of OTA in *A.ochraceus.*


Figure 6: Alignment of "AoKS1" and "A.ochraceus pks"

Reference

IARC (eds) 1993. International Agency for Research on Cancer, Lyon, France, p 489.
Huff, W. E., Hamilton, P.B. 1979. Jornal of Food Protection, 42, 815-820.
Harris, J.P., Mantle, P.G., 2001. Phytochemistry 58, 709–716.
O'Callaghan, J., Caddick, M.X., Dobson, A.D.W., 2003. Microbiology 149, 3485–3491.

Characterization of various PKS genes included in secondary metabolites biosynthesis pathway in Aspergillus westerdijkiae NRRL 3174

Nafees Bacha, Florence Mathieu Thierry Liboz and Ahmed Lebrihi
Université de Toulouse, Département Bioprocédés et Systèmes Microbiens. Laboratoire de Génie Chimique, UMR 5503 (CNRS/INPT/UPS). ENSAT-INP de Toulouse, 1, Avenue de l'Agrobiopôle, B.P. 32607, 31326 Castanet-Tolosan Cedex 1, France
E-mail address: lebrihi@ensat.fr

Introduction

Fungal polyketide metabolites are a major potential source of novel bioactive compounds. The wide spectrum of acticvity of polyketides makes them economically, clinically and industrially the most sought after molecules. *Aspergillus westerdijkiae* is a filamentous fungi capable of producing several economically important polyketides, including ochratoxine A (OTA), mellein, hydroxymellein, penicillic acid, asperlactone and iso-asperlactone. The biosynthesized of these metabolites are biosynthesized by a group of modular enzyme system known as polyketide synthases (PKSs) (Bentley & Bennett, 1999; Hopwood & Sherman, 1990).
Herein, we describe the characterization of three PKS genes i.e. *aoks1*, *aolc35-12*, *aomsas* and *aolc35-6* in *A. westerdijkiae*.

Disruption of target genes and complementary mutants formation

To functionally characterize each of the target gene various transformation vectors were generated. By using protoplasts based insertional inactivation technique, each of target gene was disrupted in the genome of *A. westerdijkiae* (Fig. 1) to obtained *aoΔks1 aoΔlc35-12*, *aoΔmsas* and *aoΔ lc35-6* mutants. The totality of the target genes were amplified and were used for the formation of genetically complemented mutants.

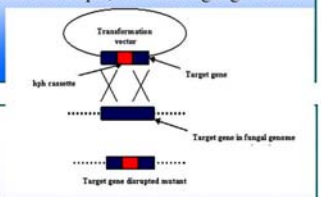
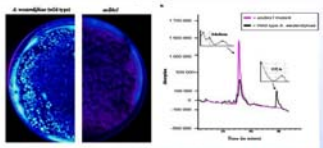


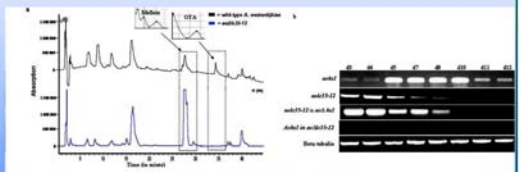
Figure 1: Disruption of target gene

Characterization of aoks1, aolc35-12, aomsas and aolc35-6 genes

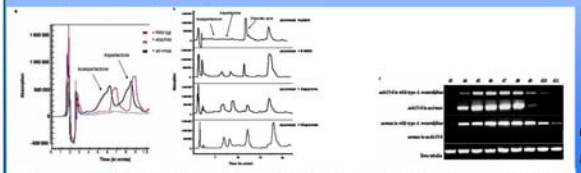
Inactivation of *aoks1* gene resulted into the disruption of ochratoxine A characteristic flurescence in aoΔks1 mutant, when observed under UV light (Fig. 2a). This has been further confirmed by analyzing the HPLC profiles of wild type *A. westerdijkiae* and *aoΔks1* (Fig. 2b).


Figure 2: Analysis of the wild type and aoΔks1 mutant (a) under UV light and (b) HPLC profile

Disruption of *aolc35-12* gene also abolished the biosynthesis of ochratoxine A in *aoΔlc35-12* mutant (Fig. 3a). Expression of *aolc35-12* gene in *aoΔks1* mutant showed that *aolc35-12* is not the ochratoxine A biosynthesis gene, but control the expression of *aoks1* gene (Fig. 3b).


Figure 3: Profile of wiled type A. westerdijkiae and *aoΔks1* mutant
(a) secondary metabolites and (b) expression

Insertional deactivation of aomsas gene revealed that this gene is involved in the biosynthetic pathway of two important polyketides asperlactone and iso-asperlactone (Fig. 4a).

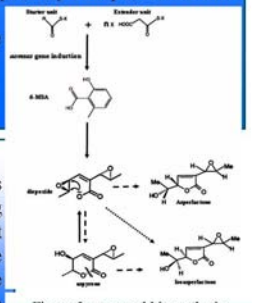

Figure 4: HPLC profile of secondary metabolites (a) after transformation and (b) after chemical complementation (c) profiles of *aomsas* and *aolc35-6* genes

Chemical complementation by the addition of 6-methylsalicylic acid,diepoxide and aspyrone revealed that these compounds are intermediates in the biosynthetic pathway of asperlactone and iso-asperlactone (Fig. 4b).

The gene "*aolc35-6*" was very close to the group of hybrid between PKS and non-ribosomal peptide synthase (NRPS). Disruption of *aolc35-6* halted the biosynthesis of three important polyketide i.e. penicillic acid, asperlactone and iso-asperlactone". This gene was found to be a regulatory gene which control expression of *aomsas* (Fig.4c) and an other unknown gene important for penicillic acid biosynthesis.

Conclusion

Atoui et al., (2006) identified KS domain fragments of several PKS genes in *A. westerdijkiae*. We characterized four of these PKS genes and identified that these genes encode important polyketide metabolites in this fungi. Two PKS genes aoks1 and *aolc35-12* are controlling the biosynthesis of ochratoxine A. Among these genes *aolc35-12* is not a biosynthesis gene but a gene that regulate the expression of *aoks1*. Further *aomsas* is a methylsalicylic acid synthase (MSAS) type PKS gene that is important for the biosynthesis of asperlactone and iso-asperlactone. 6-methylsalicylic acid, diepoxide and aspyrone are intermediates of asperlactone and iso-asperlactone. Expression of *aomsas* gene was found to be controlled by a hybrid PKS-NRPS gene i.e. *aolc35-6*.


Figure 5: proposed biosynthetic scheme of Asperlactone and isoasperlactone

References

Atoui A, Dao. P, Mathieu F, Lebrihi A. 2006. Mol Nutr Food Res 50: 448-493; Bentley R, Bennett J. 1999. Annu Rev Microbiol 53: 411-446; Hopwood D, Sherman D. 1990. Annu Rev Genet 24: 37-66 .

Résumé

*Aspergillus westerdijkiae*m qui est récemment démembré d'*A. ochraceus* est un producteur principal de plusieurs composés de type polycétone d'importance économique. Ces composés incluent l'ochratoxin A, mellein, l'acide penicillique, asperlactone et l'isoasperlactone et quelques intermédiaires comme l'acide 6-methylsalicylique et l'acide orsellinique. La biosynthèse de ces metabolites est catalysée par un groupe d'enzymes connues comme la polycétone synthases (PKSs). Ce travail a été visé pour cloner et a caractérisé fonctionnellement les différentes genes des PKS i.e. *aoks1*, *aolc35-12* et *aomsas*, et de genes de polyketide synthases-non ribosomal peptide synthase (PKS-NRPS) i.e. *aolc35-6*, chez *A. westerdijkiae.* Ces gènes ont été inactivés par l'insertion du géne d'hygromycine B phosphotransferase d'*Escherichia coli* dans le génome *d'A. westerdijkiae*, pour obtenir les mutantes *ao*Δ*ks1*, *ao*Δ*lc35-12*, *ao*Δ*msas* et *ao*Δ*lc35-6*.

Les mutants *ao*Δ*ks1* et *ao*Δ*lc35-12* ont été trouvés déficients dans la biosynthèse d'ochratoxin A, mais produisaient encore mellein. À notre connaissance, c'est la première fois que nous avons caractérisé les gènes impliquées dans la biosynthèse d'OTA, sachant que mellein, qui était proposé dans la littérature comme un intermédiaire, joue a cune role dans la biosynthesis de l'OTA. Ensuite le mutant *ao*Δ*msas* n'a pas seulement perdu la capacité de produire isoasperlactone et asperlactone, mais aussi il ne produit pas l'intermédiaire acide 6-methylsalicylique. Basé sur les expériences de la caractérisation génétique et de complémentation chimiques, nous avons proposé un shéma hypothétique de la biosynthèse d'asperlactone et isoasperlactone dans lequel l'acide 6-methylsalicylique, diepoxide et aspyrone jouent le rôle d'intermédiaires. La techniques de gène knock-out et de la reverse transcription PCR (RT-PCR) ont montré que seulle gène de type PKS-NRPS « *aolc35-6* » identifié chez *A. westerdijkiae* codant pour un intermédiaire inconnu(s) qui pourrait inciter l'expression de gène *aomsas* et un gène impliqué dans la biosynthèse d'acide orsellinique et d'acide penicillique.

Mots clé: *Aspergillus westerdijkiae*, polycétone synthase gènes, la caractérisation génétique, genome walking, transformation, complémentation chimique, ochratoxine A, l'acid penicillique, aspyrone, l'acid 6-methylsalicylique, isoasperlactone et asperlactone.

Abstract

*Aspergillus westerdijkiae*m which is recently dismembered from *A. ochraceus*m is the principal producer of several economically important polyketide metabolites. These metabolites include ochratoxin A, mellein, penicillic acid, asperlactone and isoasperlactone and some intermediates like orsellinic acid and 6-methylsalicylic acid. The biosynthesis of these metabolites is catalyzed by a group of enzymes known as polyketide synthases (PKSs). This work was aimed to clone and functionally characterized various PKS i.e. *aoks1, aolc35-12* and *aomsas,* and polyketide synthases-non ribosomal peptide synthase (PKS-NRPS) genes i.e. *aolc35-6,* in *A. westerdijkiae.* These genes were inactivated by the insertion of *Escherichia coli* hygromycin B phosphotransferase gene in the genome of *A. westerdijkiae* to obtain *ao*Δ*ks1, ao*Δ*lc35-12, ao*Δ*msas* and *ao*Δ*lc35-6* mutants.

*ao*Δ*ks1, ao*Δ*lc35-12* mutants were found deficient in ochratoxin A biosynthesis but are still producing mellein. To our knowledge, we for the first time characterized a gene involved in OTA biosynthesis, with the information about mellein which was proposed in the literature to be an intermediate OTA.Further *ao*Δ*msas* mutant not only lost the capacity to produce isoasperlactone and asperlactone but also the intermediate nature product 6-methylsalicylic acid. Based on the genetic characterization and chemical complementation experiments, we have proposed a hypothetical pathway mentioning that 6-methylsalicylic acid, diepoxid and aspyrone are intermediates of isoasperlactone and asperlactone. Gene knockout technique and reverse transcription PCR (RT-PCR) shown that the only PKS-NRPS gene *aolc35-6* so far identified in *A. westerdijkiae* encoding certain unknown intermediate(s) which induces the expression of *aomsas* gene and a gene involved in the biosynthesis of orsellinic acid and penicillic acid.

Key words: *Aspergillus westerdijkiae*, polyketide synthase genes, genetic characterization, genome calking, transformation, chemical complementation, ochratoxin A, penicillic acid, aspyrone, 6-methylsalicylic acid, isoasperlactone, asperlactone,