# Gene deletion studies in analysis of the role of cytochrome P450 in flaviolin metabolism in Streptomyces coelicolor.

**Sanchez, Lara Martin**

# Gene deletion studies in analysis of the role of cytochrome P450 in flaviolin metabolism in *Streptomyces coelicolor.*

Lara Martín Sánchez

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P.O. Box 1346
Ann Arbor, MI 48106 – 1346

## ABSTRACT

Cytochromes P450 monooxygenases play important roles on oxidative decoration of secondary metabolites in Streptomycetes.

Cytochrome P450 158A2 (CYP158A2) and CYP158A1 of *Streptomyces coelicolor* A3(2) are two of the 18 CYPs that comprise the CYP complement or "CYPome" of *S. coelicolor* A3(2). CYP158A2 catalyzes an unusual oxidative C-C coupling reaction to polymerize flaviolin (a red pigment) into three isomers of biflaviolin and one triflaviolin which are pigments responsible for UV radiation protection in *Streptomyces*. Although located in a different part of the chromosome, CYP158A1, sharing 61% aminoacid identity with CYP158A2, has also been shown to produce almost identical flaviolin-derived products.

The gene *sco1207* encoding for CYP158A2 was previously deleted and this mutant showed no changes on phenotype with respect to the wild type. Given that the production of the same flaviolin-derived compounds can be achieved as well by CYP158A1, this CYP is thought to be the substitute for CYP158A2 in the biosynthesis of those pigments, enforcing the mechanism for UV protection in these organisms.

In order to investigate functional redundancy between CYP158A1 and CYP158A2, PCR-based targeted mutagenesis was carried out to delete the gene *sco6998* encoding for CYP158A1 in a wild type strain (to create a single mutant) and in a strain with the gene for CYP158A2 already deleted (to create a double mutant for both genes). Phenotype observation of the mutants and metabolomic studies by mass spectrometry analyses were carried out in order to identify and quantify the amount of flaviolin in every mutant compared to the parental strain. Furthermore UV sensitivity assays were performed to measure the effect of the lack of those pigments.

The results obtained will shed light in the understanding of the function of such valuable and diverse proteins as are the CYPs, and highlight the importance of this pathway in the production of novel secondary metabolites with important biological activities.

# DECLARATIONS AND STATEMENTS

DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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# CONTENTS

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## ABBREVIATIONS

| | |
|---|---|
| bp – | base pairs |
| CDA – | Calcium-dependant Antibiotic |
| CPR – | Cytochrome P450 Reductase |
| CYP – | Cytochrome P450 |
| DHN – | Dihydroxy-naphtalene |
| DIG – | Digoxigenin |
| DMSO – | Dimethyl Sulfoxide |
| EDTA – | Ethylenediaminetetraacetic Acid |
| EI – | Electron Ionization |
| ESI – | Electrospray Ionization |
| FNQ – | Furanonaphtoquinone |
| GC – | Gas Chromatography |
| HPLC – | High Performance Liquid Chromatography |
| HPQ – | Hexahydroxyperylene Quinone |
| LC – | Liquid Chromatography |
| MS – | Mass Spectrometry |
| m/z – | Mass-to-charge ratio |
| NBT/BCIP – | Nitro Blue Tetrazolium/5-Bromo-4-chloro-3-indolyl phosphate |
| NMR – | Nuclear Magnetic Resonance |
| PKS – | Polyketide Synthase |
| ORF – | Open Reading Frame |
| SDS – | Sodium Dodecyl Sulphate |
| SSC – | Sodium Chloride Sodium Citrate |
| TAE – | Tris-Acetate-EDTA Buffer |
| THN – | Tetrahydroxy-naphtalene |
| THNS – | Tetrahydroxy-naphtalene Synthase |
| TIC – | Total Ion Chromatogram |

# 1 INTRODUCTION

## 1.1 CYTOCHROMES P450

### 1.1.1 Generalities

Cytochromes P450 (CYPs) are a superfamily of haemoproteins which are widely distributed in nature. They owe their name to the fact that they produce a peak at 450 nm when they are reduced with sodium dithionite in presence of carbon monoxide (Omura and Sato, 1962). Many studies on these enzymes have been carried out in humans due to their importance in the metabolism of xenobiotic drugs. They were first identified in 1958 by Klingenberg *et al.* in mammalian liver microsomal samples and since then multiple studies have targeted these enzymes in all kind of organisms (Kelly *et al.*, 2003).

Diversity is one of the most remarkable features of this superfamily. Members of this superfamily can be found in prokaryotes and eukaryotes. Humans have around 57 genes for these proteins while *Drosophila melanogaster* has 90, the plant *Arabidopsis thaliana* has 257 and the bacterium *Escherichia coli* has none (Kelly *et al.*, 2003). Not only are there differences in the number of genes between the organisms but also in structure, function and metabolism. There are only a few conserved features between organisms that determine the identity of this superfamily.

- The average protein fold shows a triangular prism structure.
- A few conserved amino-acid domains can be found. The only amino-acid absolutely conserved is the cysteine ligand to the prosthetic haem. Other invariant residues in most CYPs are arginine and glutamic acid within the *EXXR* domain in K-helix (Rupasinghe *et al.*, 2006). A threonine is frequently found in the I-helix associated with oxygen activation and electron transfer (Kelly *et al.*, 2003).

These enzymes are classified into families and subfamilies on the basis of amino-acid identity. Thus, one particular enzyme belongs to a family when shares at least 40 % of amino-acid sequence identity with members of that family or belongs

to a subfamily when the identity percentage is 55% or higher. A nomenclature has been established for these enzymes by Dr. David Nelson (Cytochrome P450 homepage, http://drnelson.utmem.edu/CytochromeP450.html). The families are assigned with a number, followed by a letter which corresponds to the subfamily, followed as well by another number which indicates the order of discovery of that particular enzyme. For each group of organisms a range of numbers has been set: from 1 to 49 for animals, 51 to 69 for lower eukaryotes, 71 to 99 for plants and from 101 for bacteria. However, new CYPs are being discovered as new data is being obtained from the complete sequence of genomes, therefore lower eukaryotes families are being expanding from CYP501 and plant families from 701 (Kelly *et al.*, 2003).

Eukaryotic CYPs are found in two cellular locations, associated with the endoplasmic reticulum and mitochondrial membranes. They were once considered as liver specific proteins due to their high concentration in liver cells, however they have been found in many other locations since then (Bruno and Njar, 2007). In prokaryotes P450s are soluble proteins according to what is known so far (Werck-Reichhart *et al.*, 2000b).

CYPs can be classified on the basis of several criteria. According to the kind of electron transfer system and redox partners they use, there are at least 3 classes of CYPs (Ortiz de Montellano, 1995):

- CYPs of Class I use an iron-sulfur protein (ferredoxin) and a ferredoxin reductase. Bacterial and mitochondrial CYPs are included in this class.
- The Class II enzymes are the microsomal (eukaryotic) CYPs which receive electrons from a NADPH Cytochrome P450 Reductase (CPR) which contains FAD and FMN cofactors.
- Other forms of P450s have been found which are self-sufficient and do not require electron donors. Among them fusion molecules of P450 and CPR such as CYP102A1 (also known as BM-3) (Girvan *et al.*, 2007), CYP55 (Takaya *et al.*, 2002), and CYP74 subfamily proteins which do

not require any other electron donor protein (Stumpe and Feussner, 2006), are noteworthy.

The wide distribution in the organisms of this superfamily of proteins leads to the hypothesis that they had an early origin (Deng *et al.*, 2007). It has been estimated that the first P450 emerged around 3.5 billion years ago, in an ancestral prokaryotic species, shortly after the origin of terrestrial life (Lewis *et al.*, 1998). In these reducing atmospheric conditions, P450s might have been originated to detoxify reactive oxygen species (Wickramasinghe and Ville, 1975). When the concentration of oxygen in the atmosphere dramatically increased, around 2 billion years ago, the functions of these proteins were probably redirected towards the utilisation of dioxygen for the metabolism of endogenous compounds to yield products as sterols, and xenobiotics (Lewis *et al.*, 1998). The evolution of these proteins from this moment on is mainly due to gene duplications that occurred for the first time around 900 million years ago. Each new cell lineage produced by them adapted to perform one particular function. Since then, more gene duplications and other changes have occurred and probably, the exposure of organisms to several chemical substances has accelerated the diversification process (Nelson and Strobel, 1987).

### 1.1.2 Structure of CYPs

The protein fold of the cytochromes P450 is quite conserved. The first crystal structure was obtained in 1985 for CYP101A1 from *Pseudomonas putida* by Poulos *et al.* The characteristic triangular prism structure of these enzymes has been observed in other CYPs in posterior crystal structure studies. P450s have a unique structure, as no non-P450 structure has been found to have the same fold (Kelly *et al.*, 2003). This fold is necessary for the protein to be able to carry out oxygen activation, binding of redox partners, and stereochemical requirements of substrate recognition (Ortiz de Montellano, 1995).

A scheme of this triangular prism structure is shown in the figure 1 below:

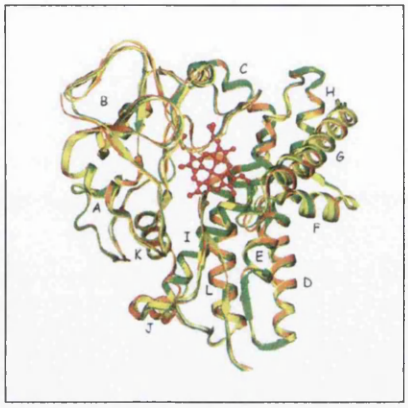


**Figure 1. CYP158A1 Structure.** The haem group is shown as the balls and sticks structure in the center of the protein (Zhao *et al.*, 2007).

Although the general fold is maintained, several structural elements present differences between organisms. The most conserved elements are the ones situated closest to the haem group, such as I and L helix while the most variable regions are those that control substrate specificity (especially B helix) (Werck-Reichhart and Feyereisen, 2000b). This structure undergoes as well conformational changes during the catalytic cycle to accommodate the substrate in the binding site (Zhao *et al.*, 2005;Zhao *et al.*, 2007) .

The following are structural elements with mechanistic or structural importance which can be found generally in all the CYPs (Lewis *et al.*, 1998):

- A 10 amino-acid motif at the start of the L-helix, including the cysteine ligand to the haem iron. This motif usually takes the form *FxxGxxxCxG* where *F* is Phenylalanine, *G* is Glycine, *C* is Cysteine and *x* is any amino-acid.
- A tetrapeptide in the K helix which takes the form *ExxR* where *E* is glutamate, *R* is arginine and *x* any amino-acid.
- A frequent threonine residue in the I-helix associated with oxygen activation.

### 1.1.3 Function and mechanism of action

Cytochrome P450-dependent monooxygenases are haem-thiolate proteins that contain an iron-protoporphyrin IX center coordinated to a cysteine thiolate. The main function of these enzymes is the monooxygenation of a wide range of

compounds (Ortiz de Montellano, 1995). This reaction requires the activation of molecular oxygen which is carried out by the transfer of electrons from the reduction of NAD(P)H by a short electron-transport chain (Werck-Reichhart *et al.*, 2000b).

The activation of molecular oxygen occurs at the iron center, where the two atoms of oxygen in the molecule are separated. One of the atoms is incorporated into a wide variety of biological substrates and the other one is transformed into water (Ortiz de Montellano, 1995;Zhao *et al.*, 2005). The general catalytic cycle of these enzymes is shown in the next scheme:

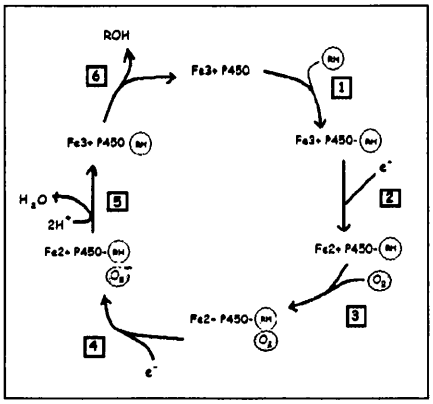


**Figure 2. The catalytic cycle of CYPs.** Steps: 1, substrate binding. 2, electron transfer. 3, dioxygen binding. 4, second electron transfer. 5, protonation and splitting of the oxygen-oxygen bond, with release of water. 6, reaction of $FeO^{3+}$ with substrate to form product, and product dissociation.

The substrate binds to the enzyme through the active site which is the haem group in the center of the protein. The enzyme needs now to bind molecular oxygen to perform its activation. In order to reduce ferric cytochrome P450 to a ferrous form, an electron from the reduction of NADPH is transferred to the enzyme by an associated reductase. In this form, the cytochrome P450 is able to bind oxygen. The acceptance of another electron and posterior protonation leads to the cleavage of the O-O bond and the release of water. The remaining intermediate transfers the oxygen to the substrate to form the oxygenated product complex. The cycle is completed when this product is dissociated (Zhao *et al.*, 2005).

These enzymes catalyse a wide array of chemical reactions, such as epoxidations, c-hydroxylations, oxygenation, aromatic dehalogenation, reductions,

oxidative rearrangements and oxidative coupling reactions (Ortiz de Montellano, 1995;Zhao *et al.*, 2005;Lamb *et al.*, 2007).

Further to their well-known role in detoxification of xenobiotic drugs and toxic chemicals, CYPs are also involved in the development of strategies for deterrence and attraction, as well as in bioremediation, being responsible for biotransformation of diverse compounds. They have the ability to produce more diverse toxic metabolites, and at the same time they possess mechanisms to detoxify them. Not only are these proteins important in secondary metabolism pathways, as in oxidative tailoring of antibiotics and other bioactive compounds, but they are also interestingly involved in the biosynthesis of primary metabolites such as sterols, steroids, fatty acids and prostaglandins. They are also potential drug targets important for antifungal and anti-protozoan agents (Kelly *et al.*, 2003;Kelly *et al.*, 2006).

Their role in the oxidative modification of diverse compounds to provide them biological activities is gaining great importance nowadays due to the need of discovery of novel compounds with application in medicine and agriculture. CYPs participate in modification of polyketides, non-ribosomal peptides and terpenes to produce compounds such as antibiotics, anti-oxidants, anticancer drugs, or antifungal agents amongst others (Ringer *et al.*, 2005;Ro *et al.*, 2006;Butz *et al.*, 2008).

## 1.2 STREPTOMYCETES

### 1.2.1 Introduction

Streptomycetes are gram-positive soil filamentous bacteria which are ubiquitous in nature. They produce the vast majority of the natural antibiotics used in human and veterinary medicine as well as in agriculture nowadays and other classes of biologically active secondary metabolites such as antiparasitic agents, immunnosuppresors, or herbicides. These organisms are responsible of several transformations of xenobiotics, and have an important role in soil biodegradation by

decomposing recalcitrant polymers thanks to the extracellular enzymes they produce (Kieser *et al.*, 2000).

They have a complex life cycle, similar to fungi's, which involves formation of two different kind of typical mycelia (aerial and substrate) and sporulation. The cycle starts when a spore finds the necessary soluble nutrients in the medium and germinates. Once they have adapted to the existent conditions, they grow exponentially as branching hyphae filaments within the medium. When environmental conditions are unfavourable, such as shortage of nutrients or other environmental stress, the so called stationary phase starts. This phase is characterised by the reduction of cell proliferation and the production of aerial mycelium. (Chakraburtty and Bibb, 1997;Hesketh *et al.*, 2007).

The production of aerial mycelium is achieved at the expense of the substrate mycelium. Part of the latter is broken and nutrients are released which are used for the formation of the aerial hyphae. As well, the tips of these hyphae undergo multiple cell divisions to generate a string of unigenomic compartments which will become the spores (Chater, 2001;Hesketh *et al.*, 2007). It is also in this phase of growth, when they produce different compounds that will permit them to survive in such an inhospitable medium as soil. These products are of very different natures such as extracellular enzymes which allow them to use non-soluble nutrients, or secondary metabolite like antibiotics, to get rid of competitors, or pigments to protect themselves against UV irradiation (Chater, 2006). Figure 3 shows a scheme of this cycle.

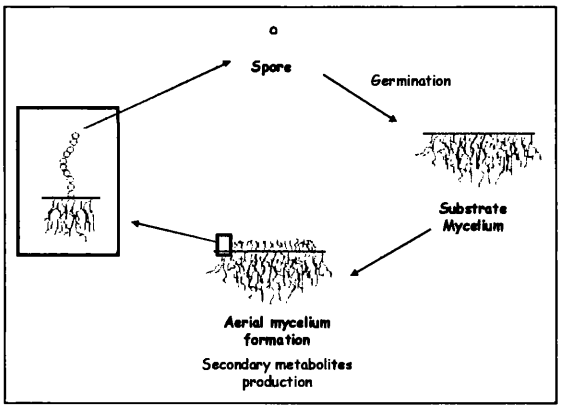


**Figure 3. Streptomyces Life Cycle.**

### *1.2.2 Streptomyces coelicolor*

*Streptomyces coelicolor* is the most genetically studied member of the genus. It is a model organism for *Streptomyces* which synthesizes a wide array of secondary metabolites. Recently, some of these secondary metabolites of *S. coelicolor* have received attention for utilization in medicine such as prodiginines (Williamson *et al.*, 2006).

*S. coelicolor* produce several chemically diverse antibiotics. The most notable ones are actinorhodin and undecylprodigiosin as they are pigmented antibiotics. Actinorhodin, a pH indicator (turning red below a pH of 8.5 and blue above) was the first studied genetically in *S. coelicolor* A3(2). Undecylprodigiosin is a mixture of at least four prodigiosins with undecylprodigiosin and butylcycloheptylprodiginine predominating. They also produce methylenomycin, a SCP-1 plasmid encoded antibiotic, and CDA, calcium-dependant antibiotic (Kieser *et al.*, 2000).

Since the genome of the strain A3(2) was completely sequenced in 2001 by the Sanger Centre at Hinxton, Cambridge (UK), genes for novel pathways of secondary metabolism were first identified, such as genes encoding cytochromes P450 among others. A figure showing the structure of antibiotics and other secondary metabolites known or predicted to be made by *S. coelicolor* can be found in Bentley *et al.* (2002).

## 1.3 CYTOCHROMES P450 IN STREPTOMYCETES

### 1.3.1 Introduction

In Streptomycetes, CYPs seem to be involved, directly or indirectly, in secondary metabolism functions and xenobiotic transformations. Genes for these CYPs are often associated in clusters with genes encoding proteins responsible for the biosynthesis of secondary metabolites. This organization of genes is widely conserved in streptomycetes suggesting a general role of these proteins in secondary metabolism in these organisms. CYP monooxygenases accomplish reactions of polyketides, nonribosomal peptides and terpenes post-modification, contributing to

the biological properties of these compounds. Furthermore, some of these proteins have the ability to oxidise a wide array of metabolites, therefore theirs could be the responsibility for the xenobiotic transformations catalysed by these organisms (Kelly *et al.*, 2003).

Very little is known about the function of most of the CYPs in Streptomycetes. The sequence homology with other CYPs can give us an idea of the function of these proteins. However, their real function can only be confirmed by functional genomic procedures, such as targeted mutagenesis of the genes encoding for these proteins.

### 1.3.2 *S. coelicolor* A3(2) CYPome

*S. coelicolor* A3(2) possess 18 CYPs, as was revealed from the information obtained from the sequenced genome (Bentley *et al.*, 2002). This cytochrome P450 (CYP) complement or "CYPome" was described in 2002 by Lamb *et. al* (Lamb *et al.*, 2002b). In order to achieve this, the genome was subjected to bioinformatic analysis and then the putative CYPs were heterologously expressed in *E. coli* and assayed by sodium dithionite-reduced carbon monoxide difference spectrophotometry. The latter assay reveals the characteristic Soret maximum around 450 nm indicative of these proteins. Their amino-acid sequence was compared with other CYPs of related organisms. Most of them were homologous to CYPs involved in secondary metabolism, or were found next to other proteins which could be involved in secondary metabolism.

CYP51 is the only CYP found in bacteria, plants, fungi, protists and animals. It is a sterol 14-demethylase required for sterol biosynthesis and was first discovered in yeast by Kalb et al., in 1987 (Kelly *et al.*, 2003). In *S. coelicolor* a CYP51-like protein has been found with low-level homology to other CYP51. This protein, despite having sterol 14-demethylase activity, does not produce sterols and must play another role in this organism, non-essential for its growth and survival (Lamb *et al.*, 2002a). It has been recently reported that this protein belongs to the CYP170 family of proteins. This so called CYP170A1 catalyses sequential allylic oxidations of the

sesquiterpene epi-isozizaene to yield the antibiotic albaflavenone *in vitro* (Zhao *et al.*, 2008).

CYP105D5 from *S. coelicolor* shares more than 90% of amino-acid identity with CYP105D4 from *S. lividans* and 69.5% of amino-acid identity with CYP105D1 from *S. griseus*. It has been reported than the CYP105 family of proteins play an important role in biotransformation and bioremediation (Lamb *et al.*, 2002b). In fact, CYP105D4 from *S. lividans*, is encoded within the AUD4 gene cluster, which is an amplifiable element or amplifiable unit of DNA. Amplifications of DNA can be interpreted as a response to the demand of higher resistance to toxic metals or antibiotics (Schmid *et al.*, 1999). CYP105D1 from *S. griseus* has been reported to be able to catalyse transformation by oxidation of several xenobiotic compounds, such as benzopyrene, erythromycin, warfarin and testosterone (Taylor *et al.*, 1999).

CYP107 family is, as well as the CYP105 family, associated with xenobiotic and secondary metabolism (Lamb *et al.*, 2003). Three members of this family were found in *S. coelicolor*, CYP107U1, CYP107P1 and CYP107T1 although they are not linked to any secondary metabolite gene clusters.

CYP156A1 and CYP154A1 are homologous to CYPs with known function involved in secondary metabolism, such as the antibiotic tylosin biosynthesis in *S. fradiae*. They are located adjacent on the same strand which suggests polycistronic translation (Lamb *et al.*, 2002b). The structures of CYP154A1 and CYP154C1 have been determined and found to catalyse different substrates despite of their similarity in amino-acid sequence (Podust *et al.*, 2004). CYP157A1 and CYP154C1 are also located adjacent in the chromosome and are part of a so called conservon together with a sensor kinase and an ATP binding protein among other genes, which suggests as well involvement in secondary metabolism. In fact, CYP154C1 has been found to catalyse hydroxylation of precursors of macrolide antibiotics in *S. venezuelae* (Podust *et al.*, 2004). CYP105N1 is also located in an operon including a non-ribosomal peptide synthase, a thioesterase, and an ABC transporter.

CYP158A2 shares 81.4% identity with a CYP from *S. avermitilis* (CYP158A3), and both of them are in the same locus, which is adjacent to a

polyketide synthase (PKS) (Lamb *et al.*, 2002b). These CYP catalyses the coupling of two flaviolin molecules to produce dimers and trimers which are thought to be pigments for the protection against ultraviolet irradiation (Zhao *et al.*, 2005). CYP158A1 is as well similar to a CYP of *S. avermitilis*. It has been reported that these CYP also uses flaviolin as substrate to yield almost identical flaviolin derivatives, although by a different substrate binding mechanism (Zhao *et al.*, 2007).

Different and varied roles have been associated to these proteins. A project to determine the function of the 18 CYPs forming the CYPome of *S. coelicolor* A3(2) is ongoing. It is believed that many other novel interesting functions will be found (Zhao and Waterman, 2007).

### 1.3.3 Gene organization

CYP monooxygenases accomplish reactions of polyketides post-modification, contributing to the biological properties such as antibiotic potency and efficacy in Streptomycetes (Kelly *et al.*, 2003). Genes for these CYPs are frequently associated in clusters with genes for polyketide synthases (PKS) in these organisms. This organization of genes is widely conserved among Streptomycetes. A comparison of this gene organization in several actinomycetes species is shown in figure 4.

The gene *rppA* encoding a type III Polyketide synthase (1,3,6,8-tetrahydroxynaphtalene (THN) synthase) was first described in *S. griseus*. In this bacterium P450mel is the enzyme responsible for post-modification of the polyketide THN. The gene *p450mel* is associated in cluster with *rppA*. A gene with high amino-acid sequence similarity to P450mel has also been found in *S. coelicolor* (gene for CYP158A2), *Saccharopolyspora erythraea* or *S. avermitilis* but not in *S. antibioticus* (Figure 4). In *S. antibioticus* another gene, *momA* encoding for a quinone-forming monooxygenase has been found associated in cluster with *rppA*. Homologues to this gene *momA* have also been found in *S. coelicolor*, *S. avermitilis* and *S. erythraea* forming a three gene operon with *rppA* and homologues of *p450mel*.

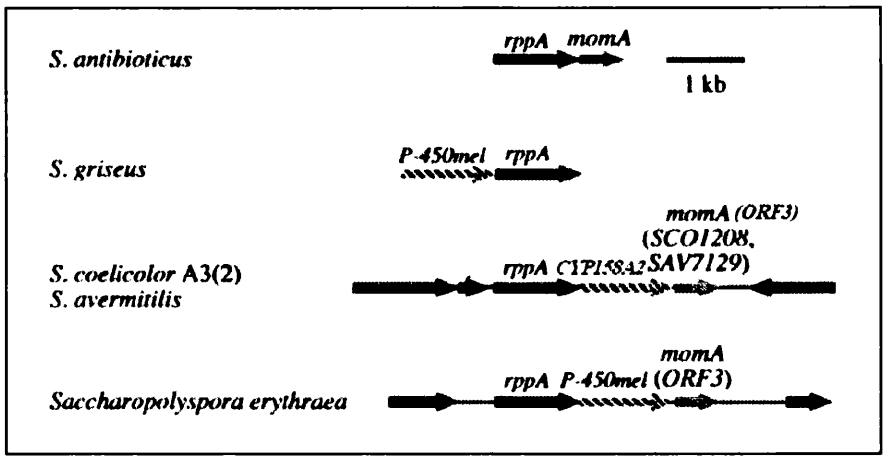


**Figure 4.** Organization of *rppA* and P450 genes in several species of actinomycetes (Funa *et al.*, 2005b).

To corroborate that the type III PKS encoded by gene *rppA* was involved in the biosynthetic pathway of pigments which provide protection against UV radiation in actinomycetes, this gene was inactivated in *S. griseus* and *Saccharopolyspora erythraea.* An albino phenotype was observed in these mutants (Cortes *et al.*, 2002;Funa *et al.*, 2005a). However, the same disruption experiments carried out with their homologous gene *sco1206* encoding for THN synthase (THNS) or *sco1207* encoding for CYP158A2, led to no changes in phenotype of the mutants (Funa *et al.*, 2005a;Zhao *et al.*, 2007).

Recently, a THNS has been identified as well in *S. peucetius* sharing 33% identity with the THNS encoded in the gene *sco1206* of *S. coelicolor* and 32% identity with RppA in *S. griseus.* This protein synthesises THN which is spontaneously oxidised to flaviolin (Ghimire *et al.*, 2008).

### 1.3.4 CYP158A2 and CYP158A1

CYP158A2 and CYP158A1 share 61.2% of aminoacid identity (Lamb *et al.*, 2002b). These two monooxygenases catalyse the oxidative coupling of two flaviolin molecules to yield pigments for the protection against UV irradiation, although by different mechanisms (Zhao *et al.*, 2007).

CYP158A2 was investigated functionally and structurally in 2005 (Zhao *et al.*, 2005). It is encoded in a three-gene operon (*sco1206-sco1208*) together with

gene *rppA* encoding a type III Polyketide synthase and *ORF3* (Izumikawa *et al.*, 2003) which presents similarity of amino-acid sequence with the gene *momA*, first described in *S. antibioticus* (Funa *et al.*, 2005b).

P450mel, also called CYP158B1, catalyses aryl coupling of THN to yield 1,4,6,7,9,12-hexahydroxyperylene-3,10-quinone (HPQ). HPQ polymerises non-enzymatically to yield brownish pigment called HPQ-melanin. These melanin-like pigments are usually biosynthesised via dihydroxyphenylalanine (DOPA) in most *Streptomyces* species, as well as in other microorganisms, plants or animals (Funa *et al.*, 2005a). THN can also be transformed into 1,8-dihydroxynaphthalene (DHN) which polymerises to produce DHN-melanin. In fungi, a type I PKS is involved in this process (Plonka and Grabacka, 2006).

CYP158A2 sharing only 46% of amino-acid sequence with P450mel catalyses the same aryl-coupling reaction. CYP158A2 uses the red-brown pigment flaviolin (2,5,7-trihydroxy-1,4-naphtoquinone, see figure 5) as substrate and produces 4 different dimeric and trimeric products by oxidative coupling.

The structure of two of these dimeric products has been characterized by Zhao *et al.* (2005) by mass spectrometry and NMR (Nuclear Magnetic Resonance) methods. They have been established as 3,8'-biflaviolin, a non-symmetrical flaviolin C-C dimer, and the symmetric 3,3'-biflaviolin. The other two products were another dimer and a trimer. These flaviolin dimers and trimers are red-brown pigments that seem to protect the bacterium from UV radiation.

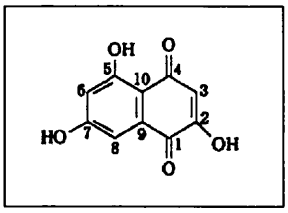


**Figure 5. Flaviolin,** 2,5,7-trihydroxy-1,4-napthoquinone.

To produce these dimeric products this enzyme binds two molecules of flaviolin in the active site. The protein has to undergo conformational changes to accommodate the two molecules at the same time. A comparison of the crystal

structure of the substrate-free protein and the substrate-complex shows how the former presents a more open conformation to allow the substrate the access to the active site (see figure 6). In this structure the F/G helices are rotated out of the active site so that the cleft reflects a gap between F helix and b-sheet 4. On the other hand, when the substrate is bound to the protein, the access channel closes. The protein undergoes significant conformational change, which represent over the 35% of the amino-acid sequence of CYP158A2 (Zhao *et al.*, 2005).

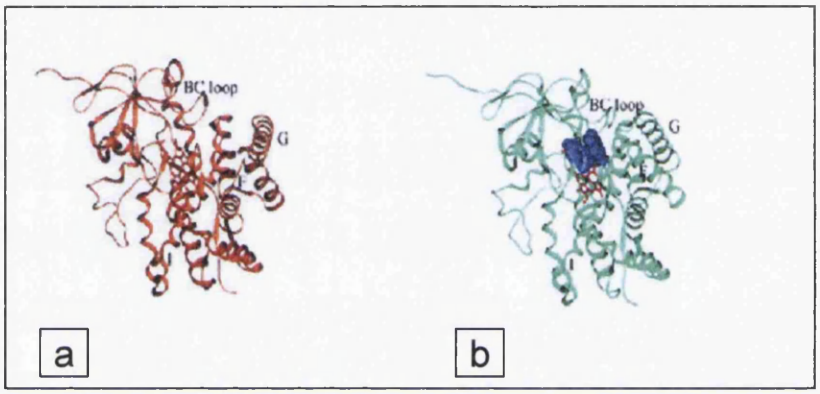


**Figure 6.** Diagram of the crystal structure of CYP158A2 in a substrate-free state (a) showing an open configuration, and when the substrate is bound (b) showing a closed configuration. The group haem is shown in red in the center of the protein. The substrate flaviolin is shown in blue. (Zhao *et al.*, 2005).

Flaviolin is the product of the oxidation of the polyketide THN, synthesised by the action of RppA from five molecules of Malonyl-CoA (figure 7). In *S. coelicolor* this reaction was proposed to be spontaneous. Later in other actinomycetes, such as *S. antibioticus* and *Saccharopolyspora erythraea* this reaction was found to be catalysed by a protein, product of gene *momA* which has been characterised as a quinone-forming monooxygenase (Funa *et al.*, 2005b). This gene *momA* has been found next to *rppA* in several actinomycetes species and it shares 58% of amino-acid sequence with ORF3 of *S. coelicolor*.

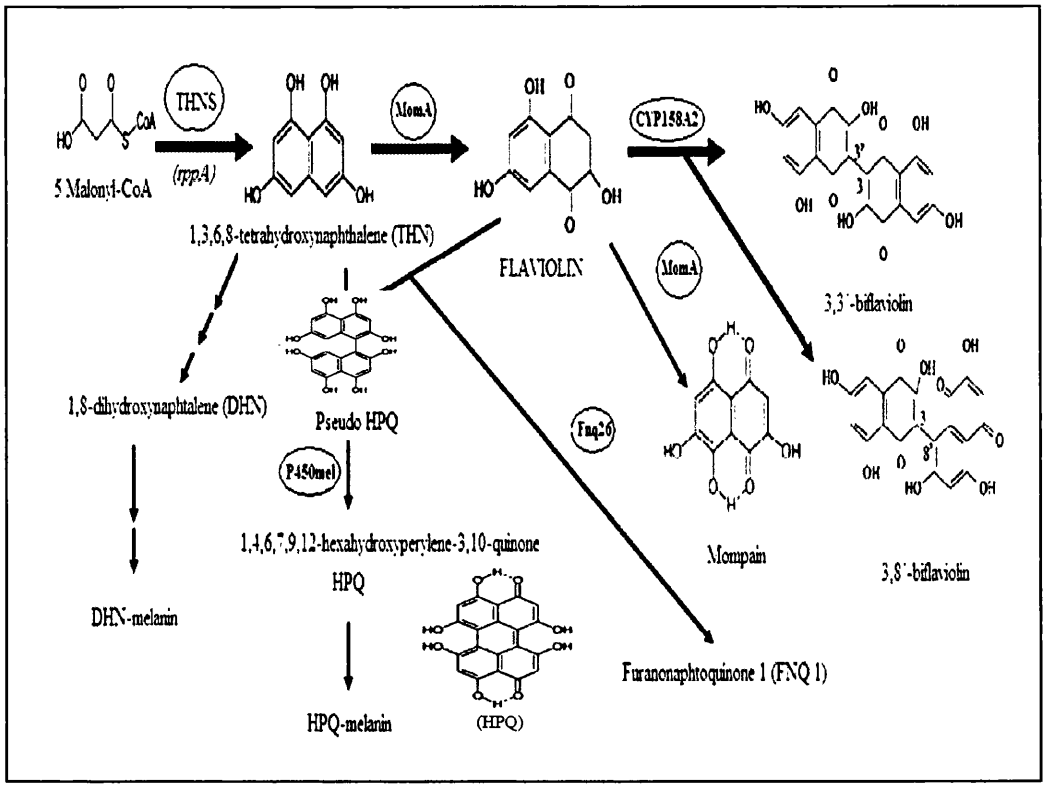


**Figure 7. Biosynthetic Pathway of flaviolin and melanin-like pigments in actinomycetes.** MomA is present in *Streptomyces antibioticus*, *S. coelicolor*, *S. avermitilis*, and *Saccharopolyspora erythraea*. P450mel, also called CYP158B1, has been found in *S. griseus*, as well as in *S. avermitilis* or *S. erythraea*. In *S. coelicolor* CYP158A2 is the cytochrome responsible of modification of flaviolin. DHN-melanin pathway occurs in fungi with a type I instead of type III PKS involved.

A homologue of *momA* has been found in *S. coelicolor* A3(2). This gene, *whiE-ORFII*, which shows similarity in amino-acid sequence with *momA*, catalyses as well the oxygenation of THN to produce flaviolin. It is located within *whiE* gene cluster, for the biosynthesis of a grey spore pigment (Funa *et al.*, 2005a).

In fungi, flaviolin has been found to be a shunt metabolite, not an intermediate but a product of the accumulation of other intermediates, of the biosynthetic pathway of DHN melanin (Bell *et al.*, 1976;Wheeler and Stipanovic, 1985;Dahiya and Rimmer, 1988;Romero-Martinez *et al.*, 2000).

Flaviolin can also be the precursor of other compounds as it has been reported for other actinomycetes (figure 7). MomA has also been reported to be involved in the modification of flaviolin to yield mompain in *S. antibioticus* (Funa *et al.*, 2005a). Derivatives of flaviolin have been found to be constituents of the diffusible red

pigment produced by *Saccharopolyspora erythraea*, in which biosynthesis a type III PKS is involved (Cortes *et al.*, 2002).

In *S. cinnamonensis* Fnq26 was described as a prenyltransferase which can catalyze *C*- and *O*- prenylations of different phenolic substrates. *fnq26* forms part of the biosynthetic gene cluster for the prenylated polyketide furanonaphtoquinone 1 (FNQ 1) in this bacterium. When using flaviolin as substrate this enzyme catalyzes a reverse *C*-prenylation, which is the formation of a C-C bond between C3 of geranyl diphosphate (GPP) and C3 of flaviolin (the normal (non-reverse) prenylation is the formation of a bond between C1 of GPP and C3 of flaviolin). It has been reported, however, that flaviolin is not the substrate of Fnq26 to produce FNQ 1, but probably an intermediate in the biosynthesis of flaviolin from Malonyl-CoA (Haagen *et al.*, 2007). In *Streptomyces* sp. strain CL190 a prenyltransferase is involved in the biosynthesis of the anti-oxidant naphterpin. This enzyme could use flaviolin and other THN derivatives as substrate *in vitro*. However, flaviolin was not the substrate of this enzyme in the biosynthesis of this anti-oxidant, and the actual substrate has not yet been determined. In *S. coelicolor* the protein HypSc has also been seen to catalyse prenylation of THN derivatives (Kuzuyama *et al.*, 2005).

CYP158A1 is encoded for the gene *sco6998* in a different part of the chromosome of *S. coelicolor* and is not organised in an operon with other genes. This CYP uses flaviolin as well as substrate to yield almost identical flaviolin derivatives. But the mechanism in which this protein couples flaviolins is not the same (Zhao *et al.*, 2007). The active site of CYP158A1 is different from that of CYP158A2. When a flaviolin is bound to the active site of CYP158A1 there is not enough space for a second one to bind in the proximal zone of the active site, as occurred with CYP158A2, therefore the two flaviolins are more separated. However, this coupling although different from that for CYP158A2 leads to the same products, 3,8'-biflaviolin and 3,3'-biflaviolin, although in a different ratio. The other two products yield by CYP158A2, another flaviolin dimer and a flaviolin trimer were not observed with CYP158A1 (Zhao *et al.*, 2007).

Flaviolin derivatives are thought to be pigments for the protection of *S. coelicolor* against UV irradiation. Disruption of the gene encoding for P450mel

(CYP158B1) a homologous of CYP158A2, in *S. griseus* increased the sensitivity of the mutant against UV irradiation (Funa *et al.*, 2005a). Mutagenesis experiments to disrupt the gene *sco1207* encoding for CYP158A2 have been carried out in order to elucidate the role of this protein in this bacterium (figure 8) (Skaug, 2006). However, pigmentation or metabolic profiling with HPLC/mass spectrometry using different extraction procedures and culture conditions did not reveal differences from the parental strain (Funa *et al.*, 2005a;Zhao *et al.*, 2007). For this reason, and given that CYP158A1 has a very high sequence similarity with CYP158A2 and both produce almost identical products by oxidative coupling of flaviolin, CYP158A1 was thought to be assuming the role of CYP158A2 in those mutants. Thus, these bacteria would have a double mechanism of protection against UV irradiation.

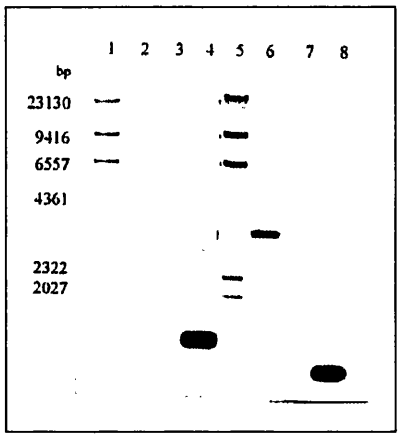


**Figure 8**. Confirmation by Southern Blot Hybridisation of the disruption of the gene *sco1207* encoding for CYP158A2. Lane 1-4: Probe: Apramycin resistance disruption cassette (1384 bp). Lane 5-8: Probe: The gene encoding for CYP158A2 (1212 bp)Lane 1 and 5: λDNA/*Hin*dIII marker; lane 2 and 6: wild type genomic DNA; lane 3: Mutant for CYP158A2 genomic DNA (hybridisation with the disruption cassette in the 3300 bp fragment; lane 4: Positive control (apramycin reistance disruption cassette); lane 7: Mutant for CYP158A2 genomic DNA (no hybridisation); lane 8: Positive control: gene encoding for CYP158A2 (Skaug, 2006).

## 1.4 FUNCTIONAL GENOMICS

### 1.4.1 Introduction

In 1989 the first project for sequencing a complete genome was programmed. An international consortium was set up in order to sequence the genome of *S. cerevisiae*. In 1995, *Haemophilus influenzae* was the first living organism to have its genome sequenced. Since then, numerous genome sequences from diverse organisms

have been and are still being published. That sequence of nucleotides contained the information of every single aspect of the biology of those living organisms. But it needed to be deciphered. The first approaches to do this consisted of figuring out the organisation of the genes and their interactions. As new data was being released new questions were arising. Transcriptomics, studies on gene expression levels, sometimes accompanied by proteomics are still shedding new light on understanding the biological processes of living organisms. A new field on functional genomics has arisen, as a new approach to try to elucidate the function of genes. This new strategy consists of studying the set of metabolites on the cell in a particular moment known as the metabolome (Oliver *et al.*, 1998;Tweeddale *et al.*, 1998).

### 1.4.2 Studies on metabolome

Functional genomics is the part of genomics which tries to assign function to the genes as well as to understand their organization and interactions. Such information can be obtained analysing the mRNA transcripts or proteins encoded by those genes and also from the set of metabolites of a biological system or metabolome (Oliver *et al.*, 1998;Fiehn, 2002).

Metabolomics, or the identification and quantification of all the metabolites of a biological system (Fiehn, 2002), emerged at the end of the 90's as a new strategy of functional genomics beside mRNA profiling (transcriptomics) and proteomics (Werner *et al.*, 2008). This new approach is important to link gene function to phenotype, as it provides detailed measurement of phenotypic responses of living organisms to genetic or environmental changes (Kiefer *et al.*, 2008).

There are a wide array of analytical methods developed to obtain information from the metabolome of an organism. These methods range from chromatography coupled to mass spectrometry, nuclear magnetic resonance (NMR) or other biophysical method such as fourier-transform infrared (FTIR) spectrophotometry. All of them are based on the separation and identification of the components of a biological sample. Metabolomic experiments usually require the combined use of some kind of chromatography with mass spectrometry to achieve high resolution results.

#### 1.4.2.1 Mass Spectrometry

Mass Spectrometry is an analytical technique which identifies the chemical composition of a sample based on the mass-to-charge ratio (m/z) of its components. (Barker, 1999) It basically involves the ionization of the molecules, separation of the ions formed according to the m/z ratio and detection in proportion of their abundance in order to produce a mass spectrum which will identify that compound in the sample.

The first step is the ionization of the molecules to yield the so called molecular ions. This molecular ion normally undergoes fragmentations. The pattern of fragmentation provides information concerning the nature and the structure of the analyte. The first ionization procedure used was the electron ionization (EI) which produces an extensive fragmentation of the molecules and covers a narrow mass range limiting this technique to small molecules. The ionization techniques have evolved since the dawn of Mass Spectrometry to cover the increasing demand of new applications such as analysis of biological compounds which have high molecular masses. Nowadays, there is a wide variety of other techniques ranging from electrospray ionization (ESI) which is used for High Resolution Mass Spectrometry and operates by creating a fine spray of highly charged droplets in the presence of an electric field, to the Matrix-assisted Laser Desorption Ionisation or MALDI technique, which provides more sensitivity minimizing the sample damage, and is suitable for proteins, and other biomolecules analyses (Hoffmann and Stroobant, 2001).

Once the ions have been produced they are separated by their m/z ratio in the analyzer. The upper mass limit, the transmission of ions and the resolution are the main characteristics of a mass analyzer which have to be considered to choose the most appropriate one according to the purpose of the analysis. The procedure in the first mass analyzers consisted of separating the ions by accelerating and focusing them into a beam. This beam was then subjected to a strong magnetic field perpendicular to its direction which produced deflection of the ions. The radius in which these ions deflect is inversely proportional to the mass of the ions. Thus, lighter ions are deflected more than the heavier ones. However, as the ionization

sources and techniques have been changing, the mass analyzers had to do so as well to meet the demands. Thus, a wide array of mass analyzers is nowadays available which perform the separation of ions in many other ways, such as quadrupoles, quadrupole ion traps, Time-Of-Flight (TOF) analyzers, or ion cyclotron resonance (ICR) analyzers (Barker, 1999;Hoffmann and Stroobant, 2001).

Finally, the ions reach the detector where their abundance is actually detected by converting the ion energy into electrical signals which are then transmitted to a computer to store and analyse the data.

To summarize, a Mass Spectrometer basically consists of an ion source, a mass analyzer, and a detector. All these devices must work under high vacuum. This is necessary to avoid unwanted ion collisions with other gaseous molecules which produce deviations in the trajectory of the ions, thus complicating the interpretation of the spectrum (Hoffmann and Stroobant, 2001).

Application of Mass Spectrometry to biological samples analysis usually requires coupling with other analytical techniques such as chromatography to separate the compounds to be analyzed first from such complex biological samples. Thus, mass spectrometry can be coupled to gas chromatography (GC/MS), liquid chromatography (LC/MS) or even to another mass spectrometer in the so-called technique Tandem Mass Spectrometry (MS/MS) which consists on at least two mass analyzers in series connected by a chamber called the collision cell (Barker, 1999).

## SUMMARY AND OBJECTIVES

The CYPome of *S. coelicolor* A3(2) was determined in 2002 (Lamb *et al.*, 2002b), and little knowledge still exist about the function of the vast majority of those proteins. The elucidation of function can be achieved by the attribution of a specific phenotype to a change on the genotype. This approach can be done by targeted mutagenesis which requires the fulfilment of directed gene mutations and observation of the behaviour and phenotype of those disrupted organisms. This is known as functional genomics and makes use of different and diverse techniques in order to characterise the mutants.

CYP158A1 and CYP158A2 are two of the 18 CYPs in the CYPome of *S. coelicolor*. These two proteins share high similarity of amino-acid sequence and it has been found that they catalyse the same reaction using flaviolin as substrate and producing almost identical flaviolin derivatives which are pigments for the protection against UV radiation, although by a different mechanism. The deletion of gene *sco1207* encoding for CYP158A2 did not produce the expected change of phenotype in the conditions tested. Given their similarity CYP158A1 was thought to be substituting CYP158A2 in the production of those pigments, thus suggesting the existence of functional redundancy, and this would explain the lack of phenotype.

The aim of this project was to achieve the deletion of the gene *sco6998* encoding for CYP158A1 in a single mutant for that gene, and in a mutant with the CYP158A2 gene already deleted to obtain a double mutant for both genes.

Observation of phenotype in the search of accumulation of pigments in the mycelium or spores, metabolic profiling by Mass spectrometry of the mutants in comparison with the parental phenotype and analysis on the effect of UV radiation in the spores survival, were the approaches performed to characterise the mutants generated.

# 2 MATERIALS AND METHODS

All media and solutions used were autoclaved or filtered with 2 μm of diameter filters and every culture or inoculation were set up under a safety cabinet to work under sterile conditions.

## 2.1 ROUTINE PROCEDURES

### 2.1.1 Transformation of *E. coli*

The next method was initially described by Hanahan (1983) and allows transformation of competent *E. coli* cells (Sambrook and Russell, 2001).

- Competent cells of *E. coli* ET12567/pUZ8002 are mixed with the cosmid containing a fragment of *S. coelicolor* chromosome with a gene disrupted. This mixture is kept on ice for 30 minutes. It is incubated then for 90 seconds at 42°C and placed in ice immediately after that for 3 minutes. This heat shock is necessary to alter the cell membranes enough to make the DNA molecules pass through and into the cell.
- To allow the expression of the antibiotic resistance, 600 μl of expression medium, LB[1] (Lennox Broth), are added to the solution and is incubated with agitation at 37°C for 60 minutes. Finally this culture was plated in LB agar (2% w/v) plates supplemented with the antibiotic for selection to allow only the transformed clones to grow.

### 2.1.2 Isolation of DNA

Isolation of DNA from bacteria requires the fulfilment of 3 steps: the breakage of the bacterial cell wall, lysis of the cell membrane and removal of proteins and other contaminants. Different methods exist that use different mechanisms and/or reagents to carry out those steps. The choice of a method depends on the quantity and the quality of DNA required among other factors.

[1] LB (Lennox Broth) Medium: Tryptone (10 g/l), Yeast Extract (5 g/l), NaCl (5 g/l), Glucose (1 g/l)

The breakage of the cell wall by chemical means is usually achieved by a lysozyme treatment. Lysozymes are enzymes that catalyze the acid base hydrolysis of linkages between N-acetylglucosamine and N-acetilmuramic acid residues in the proteoglycan of bacterial cell walls (Sambrook and Russell, 2001).

The cells can be lysed by chemical treatment with detergents that destroy the protein-lipid bilayer of the cell membrane and solubilize transmembrane proteins (Alberts *et al.*, 2008). SDS and Triton X100 are two commonly used detergents.

For the removal of proteins and other contaminants several approaches can be followed. Sometimes organic solvents such as phenol, chloroform, and isoamilic acid are used to this end. Phenol denatures proteins and when combined with chloroform this deproteinisation is more efficient. Thus, the proteins are firstly extracted with phenol:chloroform:isoamyl alcohol (25:24:1). The role of the isoamyl alcohol in the mixture is the inhibition of RNase activity. A second extraction is then necessary with chloroform:isoamyl alcohol to remove any traces of phenol in the sample. (Sambrook and Russell, 2001).

Phenol and chloroform are toxic or hazardous organic solvents which require special care to handle. Proteinase K is an alternative or sometimes complementary method to phenol extraction to remove proteins (Sambrook and Russell, 2001). This enzyme degrades most proteins such as RNases, DNases and lysozyme and eventually itself (Kieser *et al.*, 2000).

Once the solution containing DNA has been freed of contaminants, the DNA is generally precipitated with ethanol together with a sufficient quantity of cations to neutralise the negative charges of the phosphate groups of the DNA that were exposed by the alcohol. This amount of cations is usually provided by a solution of sodium acetate (Sambrook and Russell, 2001).

### 2.1.2.1 Genomic DNA

Extraction of *Streptomyces* genomic DNA was carried out by two different methods: Salting out procedure from "Practical Streptomyces Genetics" (Kieser *et al.*, 2000) and with QIAGEN DNeasy® Blood and Tissue kit.

The Salting out procedure was first described by Pospiech and Neumann (1995) and uses proteinase K to lyse the cells and chloroform and a high concentration of salts to precipitate proteins and other contaminants.

- Mycelium obtained from 30 ml of an overnight YEME[2] culture is suspended in 5 ml of SET Buffer[3] with 1 mg/ml of lysozyme. The solution is incubated at 37°C for 60 minutes.
- Proteinase K (0.5 mg/ml) and 1% SDS are then added and the mixture is incubated for 2 hours at 55°C to lyse the cells.
- To denature and precipitate proteins 2 ml of 5 M NaCl and, after letting it cool, 5 ml of chloroform are added to the mixture. Incubation continues for 30 minutes at room temperature.
- The solution is centrifuged for 15 minutes at 6000 rpm.
- The supernatant is transferred to a new tube where the DNA will precipitate after the addition of 0.6 volumes of isopropanol. The precipitated DNA is then recovered and wash with 70% v/v ethanol, then dried and finally dissolved in TE buffer[4].

The QIAGEN DNeasy® Blood and Tissue Kit was used following the protocol for gram positive bacteria. The method is based on the selective adsorption of DNA to silica-gel membranes.

10 ml of a YEME culture (incubated for around 30 hours) were centrifuged at 7500 rpm to harvest the cells. The bacterial pellet was resuspended in Enzymatic

[2] YEME: Yeast extract (3 g/l), bacto-peptone (5 g/l), mal extract (3 g/l), glucose (10 g/l), sucrose (34%), $MgCl_2 \cdot 6H_2O$ (5 mM) after autoclaving.
[3] SET Buffer: 75mM NaCl, 25mM EDTA pH8, 20mM Tris-HCl pH7.5.
[4] TE Buffer: 10mM Tris-HCl, 1mM EDTA.

Lysis Buffer[5] and incubated for 1 hour at 37°C. The rest of the protocol can be found in the DNeasy® Blood and Tissue Handbook. This kit uses proteinase K as well to lyse the cells.

### 2.1.2.2 Plasmid DNA

#### 2.1.2.2.1 Alkaline Lysis

Plasmid and cosmid DNA from *E. coli* was isolated by alkaline lysis following the protocol described by Sambrook et al (2001) slightly modified.

- A single colony of *E. coli* carrying the plasmid to be isolated is inoculated in 10 ml of LB medium[1] supplied with the antibiotics to which the plasmid provides resistance. This pre-culture is left incubating at 37°C with shaking overnight.
- 30 ml of LB with antibiotics were inoculated with 100 µl of that pre-culture and incubated overnight at 37°C with shaking.
- The culture is then centrifuged to harvest the cells and the pellet is suspended in 5 ml of Alkaline Lysis Solution I[6]. This mixture is incubated for 5 minutes at room temperature.
- 10 ml of freshly prepared Solution II[7] are then added and the mixture is incubated in ice for 10 minutes.
- 7.5 ml of ice-cold Solution III[8] are added and the mixture is incubated in ice for another 10 minutes.
- After centrifugation at 7000 rpm for 40 minutes at 4°C the supernatant is recovered and filtered by passing it through a 5 ml micropipette tip with a ball of cotton on the bottom.
- 0.6 volumes of isopropanol are then added to the filtered solution and is incubated for 15 minutes at room temperature to precipitate DNA. The solution is then centrifuged, the pellet washed with 70% v/v ethanol and then resuspended in 500 µl of TE Buffer.
- The DNA is then treated with 40µg/ml RNAse for 30 minutes at 37°C.

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[5] Enzymatic Lysis Buffer: 20mM Tris-HCl pH8, 2mM sodiumEDTA, 1.2% Triton X100, 20 mg/ml lysozyme.
[6] Alkaline Lysis Solution I: 50mM Glucose, 25 mM Tris-HCl pH8, 10 mM EDTA, 5mg/ml lysozyme.
[7] Alkaline Lysis Solution II: 0.2 N NaOH, 1% SDS.
[8] Alkaline Lysis Solution III: 3 M Potassium acetate, 5 M Glacial Acetic Acid, pH4.8

- After that the DNA solution is phenolised to remove all the proteins. 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) is added to the solution, and after centrifugation the superior phase is recovered in a new tube where chloroform:isoamyl alcohol is added to remove any rest of phenol.
- After centrifugation the DNA solution free of contaminants is precipitated with 1 ml of 100% v/v ethanol (kept at -20°C) and 50 µl of 3M sodium acetate and left at -20°C overnight.
- The next day the solution is centrifuged and the pellet washed with 70% v/v ethanol. The pellet is dried and finally resuspended in 100 µl of TE Buffer.

#### 2.1.2.2.2 Minipreparations of plasmid DNA

Small quantity of DNA was isolated for the selection of clones after transformation of *E. coli* following this protocol based on the one described by Holmes and Quigley in 1981 (Holmes and Quigley, 1981).

- A single colony of *E. coli* is inoculated in 1 ml of TB medium supplied with the antibiotic for selection. The culture is incubated overnight at 37°C with agitation.
- After centrifugation for 4 minutes at 8000 rpm the cells are recovered and resuspended in 350 µl of STET buffer[9]. 10 µl of Lysozyme (10 mg/ml) are then added and the solution is incubated for 45 seconds with intense agitation.
- Immediately after, the solution is incubated in boiling water for 45 seconds. This treatment denatures chromosomal DNA and proteins which precipitate after centrifugation for 15 minutes at 14000 rpm. They are easily removable from the bottom of the tubes with a sterile tooth pick.
- 1/10 of the volume of 3M sodium acetate and 1 volume of isopropanol are added to the supernatant obtained to precipitate DNA. After 10 minutes the solution is centrifuged for another 10 minutes and the supernatant is discarded.
- The DNA precipitated is washed with 70% v/v ethanol, dried and suspended in 30 µl of TE buffer.

[9] STET Buffer: 8% Sucrose, 0.5% Triton X100, EDTA 50mM 10mM Tris-HCl, , pH8.

### 2.1.3 Quantification of DNA

The purified DNA was quantified by spectrophotometry with a Nanodrop™ 1000 Spectrophotometer (ThermoScientific). This is a fast, reproducible and clean way to quantify DNA and it only requires 2 µl of sample.

### 2.1.4 Gel Electrophoresis

All gel electrophoresis were carried out using 0.8% w/v agarose gels. The gels were stained with SYBR® Safe to manifest the DNA bands. 1 µl of SYBR safe was added to each 10 ml of agarose gel buffer (1X TAE[10]) with 0.8% w/v of agarose, when the latter was dissolved on the buffer and before being casted. The samples were run through the gel using 1X TAE and 90 V of voltage.

### 2.1.5 PCR reactions

All PCR reactions were done using the thermostable enzyme *Taq* DNA Polymerase. The program used for these reactions was the following:

- First denaturation step: 96°C for 4 minutes
- 33 cycles
    - Denaturation: 96°C, 30 seconds
    - Annealing: 68°C, 45 seconds
    - Polymerisation: 72°C, 1 minute 45 seconds
- Final extension: 72°C, 4 minutes

*Taq* DNA Polymerase from Invitrogen and GoTaq® from Promega were used indistinctively. DMSO (dimethyl sulfoxide) was used in every reaction as enhancing agent (10% of the reaction volume). This agent facilitates strand separation disrupting base pairing.

[10] 1X TAE (Tris-Acetate:EDTA): 20 ml of 50X TAE in 1l of deionised water (50X TAE: 242 g/l Tris base, 57.1 ml/l glacial acetic acid, 100 ml/l 0.5 M $Na_2$ EDTA pH 8.0).

### 2.1.6 Restriction Enzyme Digestions

Every digestion reaction was carried out using 10 % of the appropriate restriction buffer recommended by the manufacturer for each enzyme, at 37°C, for 2 hours.

## 2.2 MANIPULATION OF *S. coelicolor* A3(2)

### 2.2.1 Media

*Streptomyces* have a complex life cycle involving the production of typically fungal aerial and substrate mycelia. They produce spores in the last steps of their life cycle as a mean to disperse. The choice of the media to use for their manipulation has to be done according to these characteristics.

MS medium[11] (Mannitol soya flour medium, first described by Hobbs et al. (1989) (Kieser *et al.*, 2000) was the solid medium used to culture *S. coelicolor*. This medium allows good sporulation. Plates were incubated at 30°C.

Cultures in liquid medium were done with YEME medium (Yeast Extract-Malt Extract Medium). It contains a high amount of sucrose (340 g/l) to allow dispersed growth of the mycelium in the medium avoiding production of compact masses commonly produced by some *Streptomyces* species (Kieser *et al.*, 2000). The flasks were incubated with agitation, which assists as well on the avoidance of mycelium lumps production, at 30°C.

### 2.2.2 Recovery of Spores

Spores were recovered from MS plates incubated for one week using a solution of 0.025% v/v Triton X100.

2 ml of 0.025% v/v Triton X100 were added to the plates, and spores were gently scraped with a sterile cotton bud to avoid lumps of agar medium given that *S. coelicolor* is an agarolytic microorganism (Kieser *et al.*, 2000). The suspension is

[11] MS, Mannitol-Soya Flour Medium: Mannitol 20 g/l, Soya Flour 20 g/l, Agar 20 g/l

recovered in eppendorf tubes and centrifuge in a microcentrifuge. The pellet is washed with 1 ml of the same solution of Triton X100. After another centrifugation the resultant pellet is resuspended in a solution with 30% v/v glycerol and 0.025% v/v Triton X100. Spores are conserved at -80°C. Some suspensions are kept at -20°C to use frequently as inoculum for culturing.

## 2.3 REDIRECT

Targeted mutagenesis for gene replacement was carried out following the method REDIRECT (Gust *et al.*, 2002). This PCR-based methodology consists of the replacement of a gene by the introduction of a disrupted cosmid in *Streptomyces* by conjugation from *E. coli*.

A disruption cassette containing a selectable marker (spectinomycin/streptomycin resistance gene) and an *oriT* region (to allow its transfer to *S. coelicolor*) is constructed and amplified by PCR, using specific primers which will introduce a sequence matching the zone adjacent to the gene to be deleted. This gene is then replaced by the spectinomycin/streptomycin cassette by recombination in the cosmid 8F11 carrying the gene for the CYP158A1. This replacement is possible introducing both the cosmid and the cassette into an appropriate host providing a high rate of recombination such as *E. coli* BW25113 carrying the plasmid pIJ790. The disrupted cosmid is cloned into a methylation deficient *E. coli* host such as ET12567 carrying the non-transmissible plasmid pUZ8002. *S. coelicolor* has a powerful methylation-restriction system which would degrade any external DNA that is introduced in the bacterium. This is solved by passaging the cosmid through this non-methylation *E. coli* strain. Given that the cosmid has an *oriT* region, can be transferred to *S. coelicolor* then by conjugation, and recombination of the cassette with the chromosome will lead to the desired gene deletion.

The strain *E. coli* BW25113/pIJ790 transformed with the cosmid 8F11 containing a fragment of *S. coelicolor* chromosome with a gene disrupted and *E. coli* ET12567/pUZ8002 transformed with that disrupted cosmid were accomplished and

provided for this work by Dr. Colin Jackson. The CYP158A2 mutant was previously achieved by Dr. Tove Skaug in this laboratory.

### 2.3.1 Purification of the PCR template

Spectinomycin cassette was obtained from the plasmid pIJ778 (Figure 9). This cassette consists of the streptomycin and spectinomycin resistance gene *aadA*, and the *oriT* of plasmid RP4 flanked by FRT sites (FLP recombinase recognition sites). It was cloned into the *Eco*RV site of pBlueScript KS(+) obtaining the plasmid pIJ778. The cassette is flanked by *Eco*RI/*Hind*III sites that allow its isolation from the plasmid.

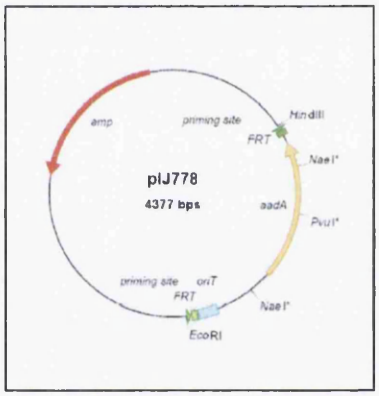


**Figure 9. pIJ778.** (Gust *et al.*, 2002)

In order to use this cassette as PCR template, it is necessary to isolate it from the plasmid to avoid a high proportion of false positive transformants (antibiotic resistant but without gene disruption transformants) that would be obtained working with the whole plasmid as a template, because of traces of covalently closed circular DNA (CCC) competing with linear PCR fragments. Therefore, the plasmid was digested with the restriction enzymes *Eco*RI and *Hind*III, and the fragment of 1425 bp corresponding to the spectinomycin cassette was purified from a 0.8% agarose gel band. The template for the PCR amplification is shown in figure 10.

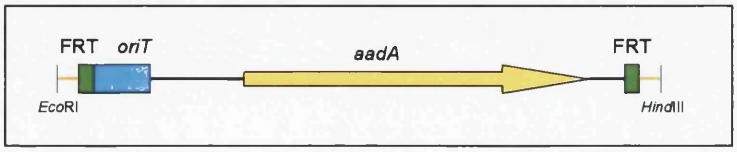


**Figure 10. Spectinomycin/streptomycin disruption cassette**. Isolated from the plasmid pIJ778.

### 2.3.2 Design of primers for PCR amplification of the spectinomycin/streptomycin disruption cassette.

Specific primers were designed and used for the PCR amplification. These primers have 19 or 20 nucleotides at the 3' end which match the end of the disruption cassette, and 39 nucleotides at the 5' end matching the sequence adjacent to the gene to be deleted (158A1). Thus, the amplified cassette is able to recombine with the chromosomal DNA of *S. coelicolor* to obtain the desired deletion (figure 11).

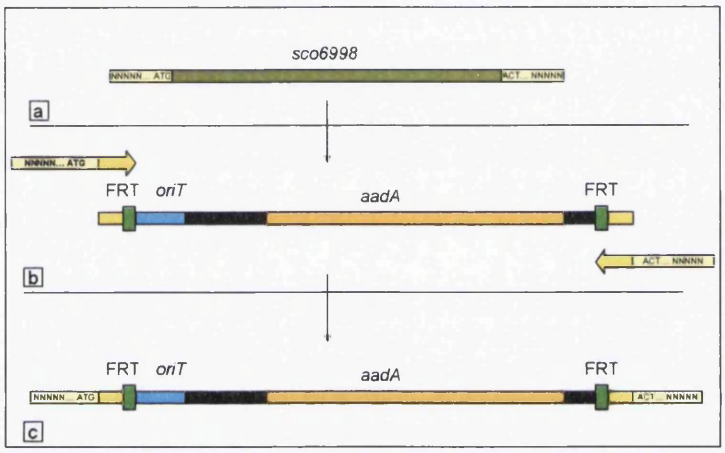


**Figure 11. a)** Gene *sco6998* encoding the CYP158A1. **b)** Design of primers. The 3' end of each primer matches the right or left end of the disruption cassette, in each case. The 5' end has the same sequence than the ends of the gene. **c)** Amplification product after PCR.

### 2.3.3 Disruption of the CYP158A1 encoding gene in the cosmid 8F11.

The disrupted cosmid, which was provided by Colin Jackson, carrying the spectinomycin cassette instead of the gene, was produced by recombination of the resistance cassette with the cosmid 8F11 carrying the CYP158A1 gene *sco6998*. This cosmid 8F11 is a Supercos 1-derived cosmid and contains a resistance gene for ampicillin.

In order to do so, both cassette and cosmid were introduced in a host which allowed high rate of recombination. This host was *E. coli* BW25113 carrying the plasmid pIJ790 which is a lambda-RED recombination plasmid containing the lambda bacteriophage genes *gam*, *exo* and *bet*.

Due to the presence of *recBCD* exonuclease DNA repair system, which destroys any invading linear DNA, many bacteria are not easily transformable with linear DNA. These three genes *gam*, *exo* and *bet* from lambda bacteriophage, encode for proteins which help to promote recombination between the viral DNA and the chromosomal DNA when the bacteriophage infects a bacterium. One of them, the product of gene *gam*, binds to the exonuclease V of the system *rec*BCD blocking it, and avoiding the degradation of the viral DNA. The products of genes *exo* and *bet* help to the formation of the recombination complex. Therefore, the presence of this λ-RED plasmid containing those genes provides high rate of recombination using linear DNA (Datsenko and Wanner, 2000;Gust *et al.*, 2004).

#### 2.3.3.1 Introduction of the cosmid and the cassette into *E. coli* BW25113/pIJ790 by electroporation

The cosmid 8F11 and the spectinomycin cassette were introduced into *E. coli* BW25113/pIJ790 by electroporation. This method of bacterial transformation consists of the application of an electrical discharge to the cells destabilizing the cell membrane and producing small pores which allows the introduction of genetic material inside them (Sambrook and Russell, 2001).

The procedure starts growing the cells (*E. coli* BW25113/pIJ790) overnight at 30°C in LB medium[12] containing chloramphenicol (25 μg/ml) to select for the pIJ790 (which contains the chloramphenicol resistance gene *cat*). 100 μl of this culture are used to inoculate 10 ml of SOB[13] medium containing 20mM $MgSO_4$ and chloramphenicol (25 μg/ml). After 3-4 hours of shaking incubation at 30°C, the cells are centrifuged and the pellet is resuspended in 10% v/v glycerol. 50 μl of this suspension were mixed with the DNA to be inserted (cosmid 8F11 DNA firstly) and electroporation was carried out. Immediately after that 1 ml of ice cold LB was added to the shocked cells and were left incubating for an hour. This culture was plated in LB agar containing the selection antibiotics (100 μg/ml ampicillin, 50 μg/ml kanamycin and 25 μg/ml chloramphenicol). Positive transformants were

[12] LB Medium: Tryptone (10g/l), Yeast Extract (5g/l), NaCl (5g/l), Glucose (1g/l).
[13] SOB Medium: Tryptone (20g/l), Yeast Extract (5g/l), NaCl (0.5 g/l), 1M KCl (2.5ml/l). After autoclaving 10 ml of 1M $MgCl_2$ are added to the medium.

selected after incubation overnight depending on the ampicillin (encoded in the cosmid) and chloramphenicol (encoded in the plasmid pIJ790) resistance.

After amplifying it by PCR with the designed primers, the spectinomycin cassette was as well introduced into *E. coli* BW25113/pIJ790 carrying as well the 8F11 cosmid by electroporation following the same procedure. After the electrical shock 1 ml of LB medium was added to the cells and incubated for an hour at 37 °C, instead that at 30°C, to promote the loss of pIJ790. When plating this culture in LB agar plates, the antibiotics used for the posterior selection of positive transformants were ampicillin, kanamycin and spectinomycin (100 μg/ml).

Homologous recombination is then expected to occur between the cosmid and the cassette. The extended cassette was constructed so that its ends match the sequence adjacent to the CYP158A1 gene in the cosmid, so recombination is possible between both. This homologous recombination does not occur in all the copies of the cosmid molecules in one cell, therefore there will be clones with both wild type and mutant cosmids (one copy of the mutagenised cosmid is sufficient to provide resistance). To select the positive transformants with the mutagenised cosmid, a colony was isolated inoculating it in LB with the selection antibiotics and incubated for 6 hours. At this time the cosmid DNA was isolated and tested by restriction analysis and PCR.

#### 2.3.3.2 Transfer of the mutant cosmids into *S. coelicolor* by conjugation.

Intergeneric conjugation is a simple way to transfer genetic material from *E. coli* to *Streptomyces*. This procedure requires the presence of vectors containing an *oriT* and transfer genes which are provided by the *E. coli* host (Kieser *et al.*, 2000). In our case this vector is *E. coli* ET12567 carrying the plasmid pUZ8002.

*S. coelicolor* carries a methyl-sensing restriction system which would degrade any external DNA that is intended to introduce in it. To avoid this, intergeneric conjugation is carried out between the non-methylating *E. coli* strain ET12567 with the plasmid pUZ8002 and *S. coelicolor*. The plasmid pUZ8002 contains a kanamycin resistance gene and lacks a *cis*-acting function for its own transfer.

Conjugation was carried out between *E. coli* ET12567/pUZ8002 and *S. coelicolor* to obtain mutants with the gene *sco6998* encoding for CYP158A1 deleted. A wild type strain was conjugated with this *E. coli* strain to obtain a single mutant for this gene, and in parallel, a *S. coelicolor* mutant with a deletion on the gene *sco1207* encoding for CYP158A2 was conjugated as well to obtain a double mutant with both genes deleted.

#### 2.3.3.2.1 Conjugation between *E. coli* ET12567/pUZ8002 containing the disrupted cosmid and *S. coelicolor*

The cosmid containing a spectinomycin/streptomycin resistance construct with the flanking DNA of the gene *sco6998* (CYP158A1) was introduced into competent cells of *E. coli* ET12567/pUZ8002 by transformation.

The transfer of the disrupted cosmid from *E. coli* ET12567/pUZ8002 to *S. coelicolor* by conjugation was achieved mixing heat-shocked spores of *S. coelicolor* with a cell suspension of the *E. coli* strain. Spores of the wild type were used to obtain the single mutant and spores from a mutant with the gene *sco1207* encoding for CYP previously deleted were used to obtain the double mutant. The procedure is detailed above.

- A pre-culture of *E. coli* ET12567 was prepared inoculating 100 µl of *E. coli* ET12567/pUZ8002 containing the gene knock-out cosmid in 10 ml of LB medium containing spectinomycin (100 µg/ml), chloramphenicol (50 µg/ml) and kanamycin (50 µg/ml). This pre-culture was incubated overnight at 37°C.

- 7 ml from this overnight culture were inoculated into 30 ml of LB with antibiotics (spectinomycin, chloramphenicol and kanamycin as above) and grown for 4-5 hours at 37°C to an $OD_{600}$ of 0.4. The cells were then washed twice with LB to remove any remaining antibiotics, by centrifuging them at 5000 rpm for 10 minutes, and then were resuspended in 3 ml of LB medium.

- While washing the *E. coli* cells, 5 µl of *S. coelicolor* spores were added to 500 µl of 2XYT broth. Heat shock was performed to these spores at 50° C for 10 minutes.

- 0.5 ml of the heat-shocked spores were mixed with 0.5 ml of *E. coli* cell suspension. After centrifugation, most of the supernatant is discarded and the cell pellet is resuspended in the 50 µl residual liquid. Dilution series were made from $10^{-1}$ to $10^{-4}$ in a total of 100 µl, and were plated in MS agar plates with 10mM $MgCl_2$ and incubated at 30°C for 16-20 hours.

- The next day, the plates were overlaid with 1 ml of water containing 0.5 mg nalidixic acid (which selectively kills *E. coli*) and 5 mg of spectinomycin. The incubation was continued at 30° C for about a week.

The same procedure was made to replace the gene encoding for CYP158A1 in the wild type strain to obtain the single mutant and in the mutant containing a deleted *sco1207* (encoding for CYP158A2) in order to obtain a CYP158A1 and CYP158A2 double mutant to compare to the existing strains.

#### 2.3.3.2.2 Screening for double cross-over exconjugants

During recombination between the disrupted cosmid and the chromosome of *S. coelicolor* a single or a double cross-over can occur. When a single cross-over happens the cassette and the rest of the cosmid are inserted in the chromosome thus providing resistance to antibiotic encoded by the cosmid and to spectinomycin. The replacement of the gene is not successful if this occurs. Double cross-over ex-conjugants, however, have the cassette inserted in the chromosome replacing the gene and are only spectinomycin resistant (Figure 12).

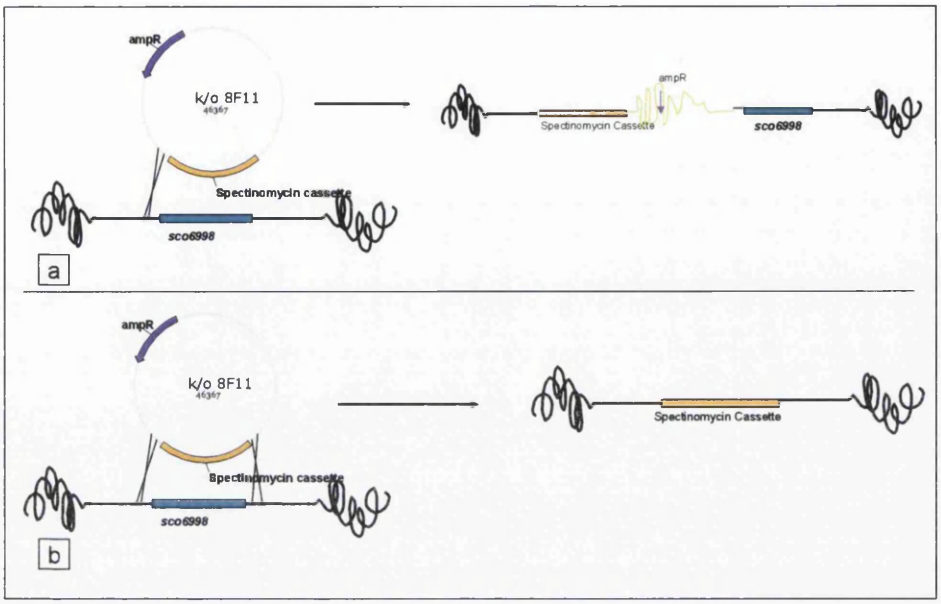


**Figure 12. Recombination between the disrupted cosmid (k/o 8F11) and the chromosome of *S. coelicolor*.** The ampicillin resistance gene encoded in the plasmid is shown as *ampR* (in purple)
**a)** Single cross-over. Insertion of the cassette and the cosmid. The gene remains in the chromosome.
**b)** Double cross-over. Replacement of the gene *sco6998* by the spectinomycin cassette.

The colonies that grew after a week in MS plates with the overlay of spectinomycin were replica-plate in MS plates containing nalidixic acid and spectinomycin with and without ampicillin (100 μg/ml). Ampicillin sensitive clones were streaked for single colonies on MS agar containing nalidixic acid (25μg/ml) and spectinomycin (100 μg/ml). The same procedure was repeated again to confirm ampicillin sensitivity: Single colonies were replica-plate in MS with nalidixic acid and spectinomycin with and without ampicillin. The confirmed ampicillin sensitive strains are then verified by PCR, Southern Blot analysis and DNA sequencing.

## 2.4 SOUTHERN HYBRIDISATION

Gene replacement was attempted to be corroborated by Southern blotting/hybridisation. This technique consists of the hybridisation of a (labelled) specific probe with the genomic DNA of the wild type strain and the mutants. For this purpose the DNA is digested with an appropriate restriction enzyme and separated by gel electrophoresis. DNA is then transferred from the gel to a nylon membrane. Two different probes were used and therefore two different membranes, each one exposed to one of the probes. One of the probes was the gene *sco*6998

encoding for the CYP158A1 and the other one was the spectinomycin cassette from the plasmid pIJ778.

### 2.4.1 Labelling of probes

The two probes used to hybridise with the genomic DNA were the gene encoding for the CYP158A1 and the spectinomycin cassette to allow total complementarity between the probe and the target DNA. They were obtained by standard PCR using specific primers. The location of each primer is shown in figure 13.

Both probes were labelled with digoxigenin (DIG) by random primed DNA labelling using the DIG High Prime DNA Labelling and Detection kit I (Roche). Digoxigenin is joined to dUTP and acts as the antigen for the antibody anti-digoxigenin. This random priming consists of the random annealing of a mixture of hexanucleotides, which would be the primers, with the probe DNA previously denatured. The klenow fragment of the DNA polymerase I from *E. coli* synthesizes then new DNA from the 3' end of the primers incorporating those dUTP DIG-conjugated. The DIG-High Prime system was the method followed to label the probes.

16 µl of DNA (about 1 µg) were denatured by heating in boiling water for 10 minutes and cooling immediately afterwards on ice. 4 µl of labelling solution (DIG-High Prime) were then added containing hexanucleotides mixture, DIG labelled DNA and Klenow enzyme. The reaction was incubated at 37°C overnight. The labelling reaction was stopped by adding 2 µl of 0.2M EDTA (pH 8).

### 2.4.2 Digestion and separation of genomic DNA

The genomic DNA of both wild type strain and the mutants was digested and separated by gel electrophoresis. The appropriate restriction enzyme to use must not cut inside the gene or the cassette. In figure 6 the restriction map of the gene and the cassette for the enzymes *Apa*I and *Bam*HI is shown. Restriction analysis was done using the online software tool Watcut, University of Waterloo, Ontario, Canada, (http://watcut.uwaterloo.ca/watcut/watcut/template.php).

*ApaI* was an appropriate enzyme to use since does not cut inside the gene nor the cassette. This enzyme provides a restriction fragment of 1783 bp which includes the whole gene and a fragment of 1984 when the cassette is inserted on the chromosome.

The enzyme *Bam*HI was as well used in several experiments. It cuts inside the gene producing two different fragments, of 1720 and 2062 bp. None of this fragments contains the whole gene. The spectinomycin/streptomycin disruption cassette has a recognition site for *Bam*HI at one of its ends. When this cassette is inserted in the chromosome replacing the gene, digestion with *Bam*HI produces a fragment of 3059 bp. The size of the DNA fragment produced with this enzyme if there was no cut inside the cassette would be 3983 bp.

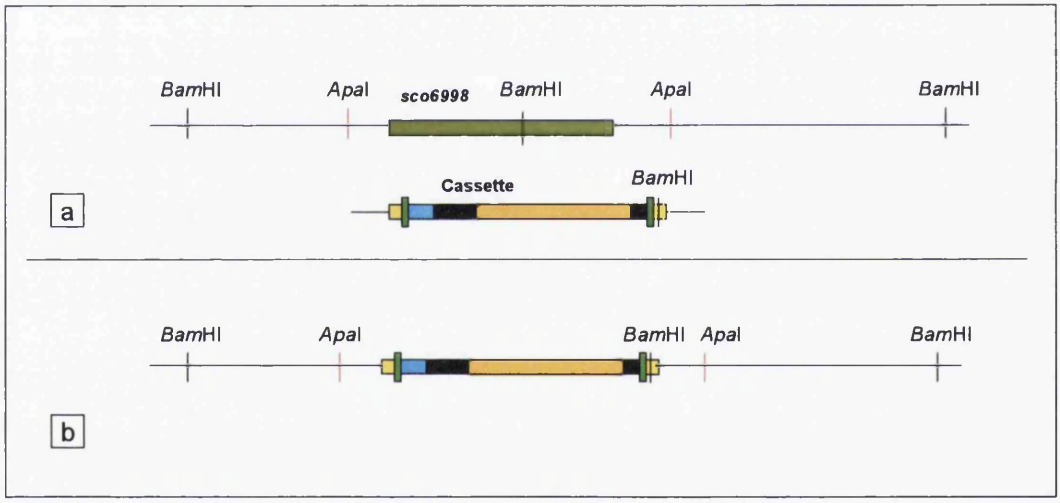


**Figure 13. Restriction map of the gene *sco6998* and the spectinomycin cassette for the enzymes *ApaI* and *Bam*HI. a)** *Apa*I produces two cuts flanking the gene producing a fragment of 1783 bp. It does not cut inside the cassette. *Bam*HI cuts inside the gene producing two fragments, of 1720 and 2062 bp. None of them contains the whole gene. It also cuts inside the cassette. **b)** When the cassette is inserted in the chromosome, digestion with *Apa*I yields a fragment of 1984 bp containing the cassette. *Bam*HI produces a fragment of 3046 bp when it cuts at the end of the cassette. If that recognition site was not there the restriction fragment would be 3983 bp.

Digestion was carried out overnight. The electrophoresis was carried out at 60 V for 2 hours. Two digestion reactions were done for each sample and the two batches were subjected to gel electrophoresis separately and transferred each one to a different membrane to hybridize with the two probes.

### 2.4.3 Capillary transfer

Once the DNA was separated by electrophoresis, the gel was subjected to several treatments to allow the transfer of the DNA to the membrane.

First of all, DNA was partially depurinated by controlled acid treatment with hydrochloric acid (250 mM HCl) for 10 minutes at room temperature and shaking. This step, however, is sometimes not necessary or even counterproductive, as the DNA can be cleaved in very small fragments that are too short to bind efficiently to the membrane (Sambrook and Russell, 2001). Therefore, both approaches were tested to see which one provided better results.

The DNA was then denatured by submerging it for 15 minutes at room temperature in alkaline denaturation solution[14] which makes DNA to break in the depurinated sites. Finally, the gel is submerged in Neutralisation buffer[15] for 15 minutes at room temperature and shaking. This neutralisation treatment is necessary to lower the pH of the gel so that the DNA can bind to the membrane.

The capillary blot was then constructed to proceed with the transfer. The gel was placed inside a recipient filled with 500 ml of 10X SSC (Sodium chloride Sodium Citrate) on an inverted plastic box. Salt concentration is an important factor in the retention of DNA by the membrane. At a concentration of 10X SSC DNA is almost completely retained (Southern, 1975). A piece of filter paper is placed behind the gel in contact with the SSC in the recipient at both sides so that the liquid will go through the blot by capillarity. The nylon membrane (Hybond-N, Amersham) was placed in contact with the membrane and above it several layers of filter and absorbent paper. A weight was placed on the top to help the transfer (figure 14). The blot was left overnight for the DNA to transfer to the membrane. Once the entire DNA was transferred, which was confirmed as there was no loading dye left in the gel, the DNA is fixed onto the membrane by UV-crosslinking. This fixation consists of the formation of a covalent bond between the amino group of the nylon of the membrane and the thymine of the DNA. Right after dismantling the blot, the

[14] Denaturation Solution: 0.5M NaOH, 1.5M NaCl
[15] Neutralisation Solution: 3M NaCl, 1.5 TrisHCl

membrane was dried at room temperature in a flat surface and then placed in an UV-Crosslinker (HL 2000 Hybrilinker, UVP laboratory products).

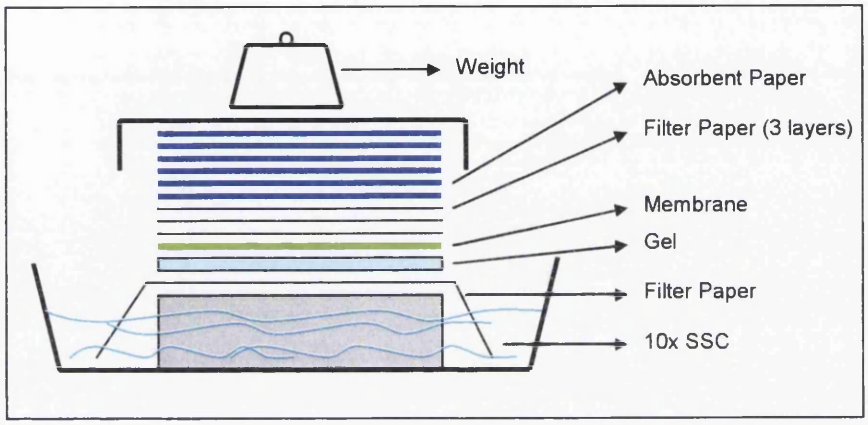


**Figure 14. Southern Blot.**

### 2.4.4 Hybridisation

Hybridisation was carried out in two different hybridisation tubes each of them carrying the membrane to be hybridised with its correspondent probe. A prehybridisation step was necessary to avoid non-specific binding blocking the zones of the membrane where there was not DNA.

The prehybridisation buffer[16] was prepared adding 1 g of *blocking reagent* (Roche) per 100 ml of solution. The membranes were inserted in a hybridization tube and prehybridisation was performed in a HL-2000 Hybrilinker hybridiser (UVP). Different temperatures and therefore different hybridisation times were tested: 68°C for 2 hours and 65°C for 3 hours. After this time the prehybridisation buffer was discarded and the hybridisation buffer was prepared adding the probes (previously denatured by heating in boiling water for 10 minutes and chilling it afterwards on ice) to the prehybridisation buffer. The membranes were left hybridising overnight at the prehybridisation temperature.

[16] Pre/Hybridisation Buffer: 5x SSC, 0.1% laurylsarcosine, 0.02%SDS, blocking reagent 1%

### 2.4.5 Stringency washes and detection

After hybridisation the membranes were washed to remove the unbound probe. The membranes were incubated in Stringency Wash Solution I[17] for 15 minutes at room temperature, two times, and after that in Stringency Wash II[18] once, for 15 minutes at room temperature.

The detection of the labelled probes was carried out by colorimetric detection by enzyme immunoassay. The membranes were incubated with an antibody anti-DIG which is conjugated with alkaline phosphatase. This enzyme uses NBT/BCIP (Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) as substrate to yield a purple precipitate.

After the stringency washes the membranes were equilibrated for 1 minute in washing buffer[19]. The zones of the membrane where there was no probe were blocked by adding 30 ml of blocking solution[20] and incubating for 30 minutes. The membranes were then exposed to the antibody solution[21] for another 30 minutes at room temperature. To remove the unbound antibody, the membranes were washed twice with washing buffer for 15 minutes and then equilibrated in detection buffer[22] for 5 minutes.

The colorimetric detection was carried out adding 200 µl of NBT/BCIP to 10 ml of detection buffer and incubating the membranes in that solution overnight in the dark. After around 12 hours, the purple precipitate was evident and the membranes were dipped briefly in distilled water to stop the reaction.

[17] Stringency Wash I (2x): 2x SSC, 0.1% SDS
[18] StringencyWash II (0.5x): 0.5x SSC, 0.1%SDS
[19] Washing Buffer: 0.3% Tween 20 in Maleic Acid Buffer (0.1M Maleic Acid, 0.15M NaCl)
[20] Blocking Solution: 1% *Blocking Reagent* [Roche] in Maleic Acid Buffer
[21] Antibody Solution: 1:3000 antibody in blocking buffer
[22] Detection Buffer: 0.1M TrisHCl, 0.1M NaCl

## 2.5 VERIFICATION OF THE MUTANTS BY PCR

PCR analyses were carried out in order to corroborate the insertion of the cassette in the genome of *S. coelicolor* and therefore the deletion of the gene *sco6998* encoding for CYP158A1. The position of the primers used for these analyses is shown in figure 15 and their sequences in table 1, annexe 2. All calculations were made according to published sequences deposited in GenBank® (http://www.ncbi.nlm.nih.gov/Genbank/index.html).

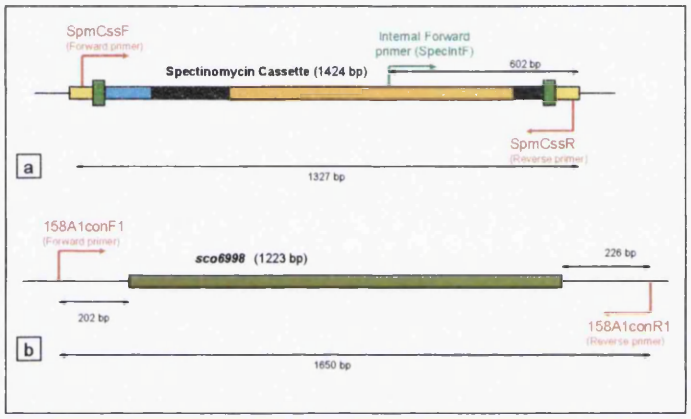


**Figure 15. Location of primers for the amplification of the gene and the cassette.** Primers are shown as red arrows. The internal test primer for the spectinomycin cassette is indicated in green. **a)** Spectinomycin cassette **b)** CYP158A1 gene.

Three different PCR reactions were done with different primers. Primers for the amplification of the gene and the cassette as well as an internal primer for the spectinomycin cassette were used to manifest their presence or absence.

The forward primer for the amplification of the gene *sco6998* (1223 bp) encoding the CYP158A1 (158A1conF1) anneals 202 bp downstream the end of the gene and the reverse primer (158A1conR1) anneals 225 bp upstream the other end (see figure 15). Thus, the PCR reaction using these primers will yield a fragment of 1650 bp. When the spectinomycin cassette (1424 bp) is inserted replacing the gene the amplification product will be 1851 bp long. Primers for the amplification of the cassette anneal 58 bp (forward, SpmCssF) and 45 bp (reverse, SpmCssR) from each end of the cassette, therefore the product obtained from this PCR reaction will be 1327 bp.

The other PCR reaction was done with a primer annealing inside the spectinomycin cassette. This primer (SpecIntF) which anneals 602 bp from the end of the cassette and the reverse primer for the gene will yield a product of 827 bp. The presence of that DNA fragment would confirm the presence of the spectinomycin cassette.

To sum up, the insertion of the spectinomycin cassette in the locus of the gene *sco6998* will be confirmed by PCR if there is DNA amplification when using primers annealing at both ends of the spectinomycin cassette or inside, and if the amplification product obtained using flanking primers for the CYP158A1 gene (*sco6998*) is 1851 bp long, corresponding to the inserted spectinomycin cassette.

## 2.6 SEQUENCING

Diagnostic DNA sequencing of the mutants was carried out in order to try a different approach to confirm the insertion of the cassette. The sequence obtained was compared with the sequence of the gene (and adjacent zones of the chromosome) and with the spectinomycin cassette. These sequences were found in GenBank® database.

Two PCR reactions using internal primers for the spectinomycin resistance and flanking primers for *sco6998* (CYP158A1 encoding gene) were undertaken. DNA was isolated from 0.8% agarose gels using the QIAquick® Gel Extraction Kit (QIAGEN). The purified DNA was quantified by spectrophotometry with a Nanodrop™ 1000 Spectrophotometer (ThermoScientific). Approximately 150 ng of DNA in solution were dried in a vacuum concentrator (Concentrator 5301, Eppendorf).

The samples were sent for analysis by Eurofins MWG Operon. An automated sequencing method was used based on chain termination sequencing method, also known as Sanger sequencing, using fluorescence labelled dideoxynucleotides.

## 2.7 STUDIES ON PHENOTYPE OF THE MUTANTS COMPARED TO THE PARENTAL STRAIN

Disruption of the biosynthetic pathway of flaviolin derivatives by deletion of the gene encoding for the CYP158A1 in a deletion mutant for CYP158A2 (double mutant) would lead to the accumulation of flaviolin which is the substrate of the enzyme. Theoretically, this would not happen to the single mutants for CYP158A1 as they still have CYP158A2 working and coupling flaviolins.

Flaviolin is a red-brown pigment. Previous studies in *S. griseus* showed that the mycelium and spores from a mutant with a deletion in the CYP158A1 homologous P450mel gene were red, unlike the wild type's (Funa *et al.*, 2005a;Funa *et al.*, 2005b).

In order to detect any phenotypical change on the mycelium or spores of the mutants, cultures in solid (MS) and liquid (YEME) medium were set for the CYP158A1 mutant, CYP158A2 mutant and the double mutant with both genes deleted.

MS plates were incubated at 30°C for 7 days and observed every day for any change on the phenotype.

30 ml YEME cultures were set at 30°C and incubated for 5 days, checking the flasks every day.

## 2.8 UV EFFECT ON SPORE SURVIVAL

Flaviolin dimers and trimers are pigments that may protect *S. coelicolor* against UV irradiation (Zhao *et al.*, 2005). Given this, and since the mutants would have the biosynthesis pathway for that pigments interrupted, spores of the wild type and mutants were exposed to short wavelength UV light to test the effect on their survival.

The protocol followed for this UV effect analysis was the one previously described by Funa et al. (Zhao *et al.*, 2005). Approximately $10^8$ spores of the wild type and the mutants were suspended in 20 ml of 10% glycerol and then irradiated with 254 nm UV light at a distance of 30 cm at different times. A Mineralight® ultraviolet lamp (UVP) was used for the experiment. 0.1 ml of an appropriate dilution of that UV-irradiated spores were plated in R5 medium[23] and incubated for 2 days. The colonies were then counted and the spore survival was calculated as the ratio between the number of colonies that appeared under UV irradiation with respect to the colonies appeared without UV treatment.

Irradiation and plating was carried out in dull light although this was not necessary given that *S. coelicolor* A3(2) cannot accomplish photoreactivation (Harold and Hopwood, 1970;Clarke and Hopwood, 1976). A search in StrepDB (Streptomyces annotation server, http://strepdb.streptomyces.org.uk) and GeneBank® database reveals that *S. coelicolor* genome contains 3 genes encoding for putative photolyases (*sco0842*, *sco0183* and *sco0184*). However, it has been reported that this organism does not show photolyase activity (Mayerl *et al.*, 1990).

## 2.9 METABOLIC PROFILING

### 2.9.1 Method of extraction

5 ml of a 4 days culture in YEME medium were harvested and its pH adjusted to 5-6 with 1M HCl or sodium bicarbonate ($NaHCO_3$) when necessary. The metabolites were extracted with an equal volume of ethyl acetate (0.5% v/v ethanol) for three times. The three extracted solutions were combined and dried with Sodium sulphate ($Na_2SO4$). The solvent was then removed in a rotary evaporator. The remaining residue was kept at -80°C until analysis.

[23] R5 medium: Sucrose 103 g/l, $K_2SO_4$ 0.25 g/l, $MgCl_2 \cdot 6H_2O$ 10.12 g/l, Glucose 10 g/l, Casaminoacids 0.1g/l, Yeast Extract 5g/l, TES buffer 5.73 g/l, 2 ml Trace Element Solution ($ZnCl_2$ 40 mg/l, $FeCl_3 \cdot 6H_2O$ 200 mg/l, $CuCl_2 \cdot 2H_2O$ 10 mg/l, $MnCl_2 \cdot 4H_2O$ 10 mg/l, $Na_2B_4O_7 \cdot 10H_2O$ 10 mg/l, $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ 10 mg/l. At time of use the following solutions were added per 100 ml of medium: 1 ml 0.5% $KH_2PO_4$, 0.4 ml 5M $CaCl_2 \cdot 2H_2O$, 1.5 ml 20% L-Proline, 0.7 ml 1N NaOH.

The samples were sent for analysis using Mass Spectrometry by the Department of Biochemistry of Vanderbilt University School of Medicine, Nashville, Tenessee.

### 2.9.2 Mass Spectrometry

Mass spectrometry was applied in the search of flaviolin in extracts of *S. coelicolor* cultures in YEME medium. To this purpose HPLC/MS analyses with electrospray ionization (ESI) were performed.

The compounds in the sample were therefore first separated by Reverse Phase HPLC according to their retention time in the column which depends on the polarity or hydrophobicity of the compounds. The more hydrophobe is a compound the longer is going to retain inside the column.

The mass spectra were obtained with a Finnigan TSQ Quantum mass spectrometer in the positive ionisation mode with electrospray voltage 4.0 kV and 300° of capillary temperature. HPLC separations were made with a YDC ODS-AQ 2.0 mm x 250 mm reversed-phase octadecylsilane column with a linear solvent gradient from 90% of 0.5% v/v formic acid/0.01% v/v trifluoroacetic acid in water (solvent A) to 100% of 0.5% v/v formic acid/0.01% v/v trifluoroacetic acid in acetonitrile (solvent B) over 20 min, followed by isocratic elution with solvent B for 10 min at a flow rate of 200 µl/min.

# 3 RESULTS

## 3.1 REDIRECT

In order to delete the gene encoding CYP158A1 in the parental strain of *S. coelicolor*, to obtain a single mutant, and in a CYP158A2 gene deletion mutant, to obtain a double mutant for both genes, a PCR-based methodology was undertaken. CYP158A2 gene was previously replaced in our laboratory using a construction carrying an apramycin resistance gene. CYP158A1 gene (*sco6998*) was replaced within the cosmid 8F11 with a construction which carries a spectinomycin/streptomycin resistance gene as a selective marker (spectinomycin cassette). The introduction of the mutation into *S. coelicolor* was achieved by conjugation with an *E. coli* strain carrying the disrupted cosmid. This cosmid contains ampicillin and kanamycin resistance gene as additional selective markers. A single cross-over between the disrupted cosmid and the chromosome of *S. coelicolor* leads to the insertion of the whole cosmid inside the chromosome providing ampicillin/kanamycin and spectinomycin resistance to the exconjugants. The gene replacement event only occurs when a double cross-over takes place during recombination, providing exclusively spectinomycin resistance.

### 3.1.1 Screening for double-cross over exconjugants

Ampicillin was the antibiotic used to screen the exconjugants for the correct antibiotic resistance phenotype. The parental strain of *S. coelicolor* was tested for growth in different ampicillin concentrations in MS medium. No growth was detected at an ampicillin concentration of 250 μg/ml, therefore this was the concentration used for the screening of double cross-over exconjugants.

104 of the exconjugant colonies for the screens for the single *sco6998* (CYP158A1) deletion and 116 for the double CYP158A1 and CYP158A2 gene deletion that grew in the MS plates with nalidixic acid and spectinomycin were plated in replica-plates with spectinomycin (100 μg/ml) with and without ampicillin (250 μg/ml). 18 out of the 104 exconjugants for the single disruption and 15 out of the 116 exconjugants obtained for the double disruption did grow in plates with

spectinomycin and did not grow in plates with spectinomycin and ampicillin. A single colony from each clone was obtained by streaking and they were replica-plated again to confirm ampicillin sensitivity.

12 exconjugants for the single disruption and 14 for the double were finally confirmed to be spectinomycin resistant and ampicillin sensitive, thus suggesting that the double cross-over took place and the replacement of the gene was successful. Those exconjugants were furthered analysed by different means to confirm the deletion.

### 3.1.2 Confirmation by Southern Blot

A DNA fragment containing the gene *sco6998* (CYP158A1) and another one containing the spectinomycin cassette used as probes were set to hybridise with genomic DNA from the mutants by Southern Blot Hybridisation. The detection of signal for the cassette probe and the absence of signal for the gene probe in those mutants would confirm the gene replacement.

Four samples were initially selected to analyse by Southern Blot Hybridisation. Samples named 3, 10, 13 and 21 were the ones chosen.

The Southern Blot analysis was unsuccessful. It was not possible to obtain enough signal for the wild type sample, therefore the results are inconclusive.

DNA concentration and quality of the wild type strain, of the mutants and the probes were measured with a nanodrop spectrophotometer at 230 nm of wavelength. The purity of the sample with respect to protein contamination was measured as the ratio 260/280. Proteins tend to absorb at 280 nm and DNA at 260. A 260/280 ratio of 1.8 is generally accepted as pure DNA. The results are shown in figure 16 and 17.

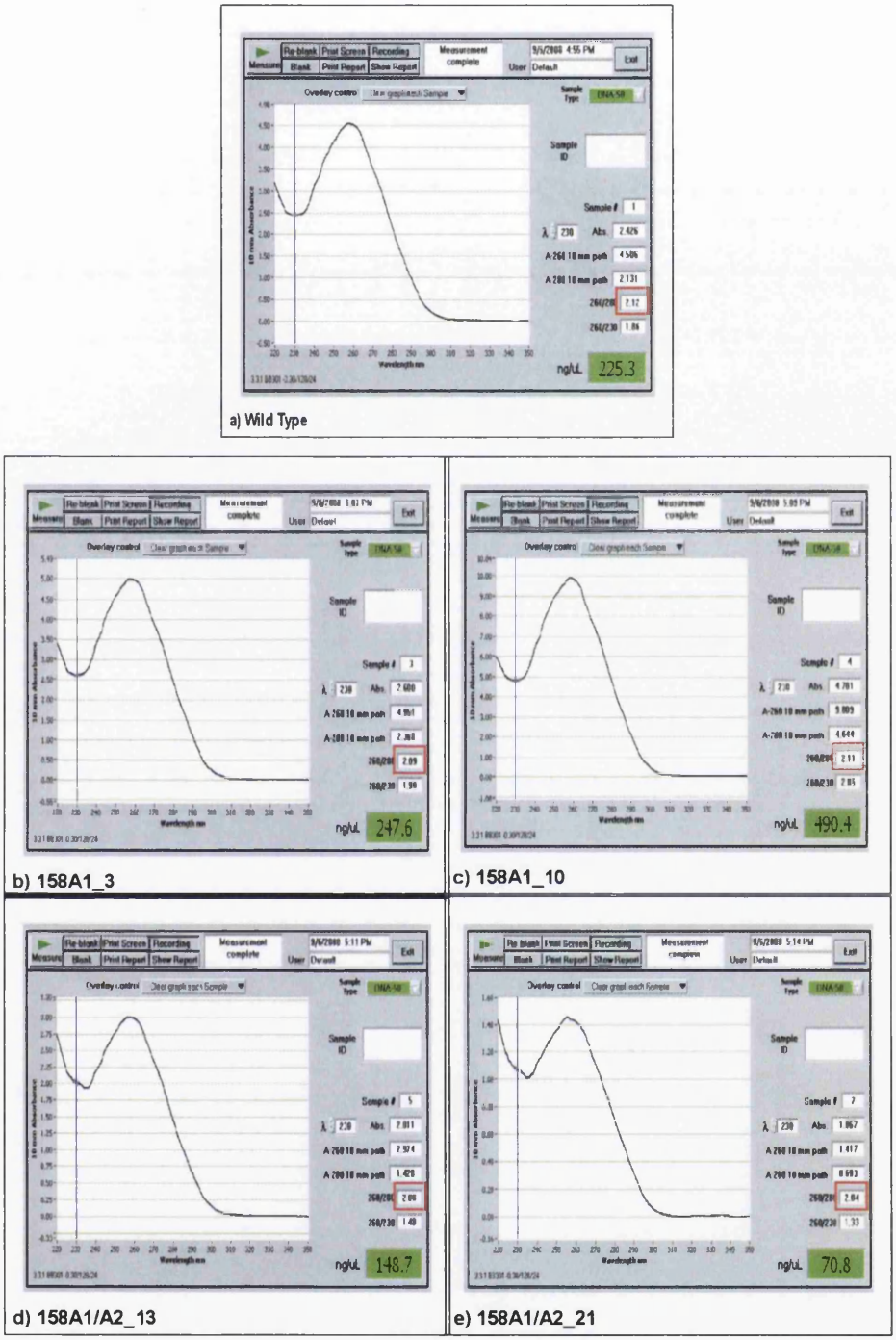


**Figure 16.** Spectra obtained with Nanodrop Data-viewer. DNA concentration (shown in the green squares in ng/µl) of the parental strain and the mutants. The purity of DNA is shown in the red square as the ratio 260/280.

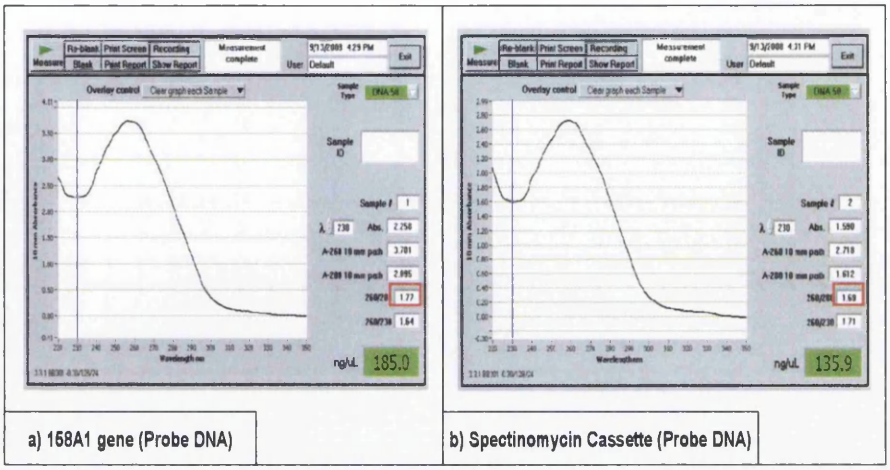


**Figure 17.** Spectra obtained with Nanodrop Data-viewer. DNA concentration and purity of the probes. a) 158A1 gene probe, b) Spectinomycin cassette probe. DNA concentration is shown in the green squares in ng/µl. The purity of DNA is shown in the red square as the ratio 260/280.

### 3.1.3 Verification by PCR

PCR reactions were done for each clone with the correct phenotype. The expected size of amplified fragments with each set of primers in the parental strain and in the mutants with the gene *sco6998* replaced by the construction is shown in table 1. Primers 158A1conF1 and 158A1conR1 amplify a DNA fragment of 1650 bp in the wild type, containing the gene *sco6998* (1223 bp). If the deletion is successful, these primers will amplify a fragment of 1851 bp, corresponding with the insertion of the spectinomycin cassette, which is 1424 bp long, 201 bp longer than *sco6998*. (See figure 15 for more detailed size fragments).

The sequences of the primers used in each PCR reaction are shown in annexe 2. Figures 18 to 20 show the bands obtained for each PCR amplification.

| **Set of primers** | **Expected size of amplified fragment (bp)** | |
|---|---|---|
| | Parental Strain | Gene Replacement |
| 158A1conF1<br>158A1conR1 | 1650 | 1851 |
| SpmCssF<br>SpmCssR | - | 1327 |
| SpecIntF<br>158A1conR1 | - | 826 |

**Table 1:** Size of amplified DNA fragments with each set of primers

One band of approximately 1800 bp was detected for samples 10 (putative single mutant for CYP158A1 gene) and 13 (putative double mutant for both CYP158A1 and CYP158A2 genes) when amplifying the DNA with flanking primers for the gene *sco6998* encoding for CYP158A1, which corresponds with the amplification of the spectinomycin cassette inserted in the place of the gene (figure 18). Samples 1, 4, 5, 6, 7, 11 (putative single mutants for *sco6998*) and 20 (putative double mutant for CYP158A1 and CYP158A2 gene) showed a band of 1600 bp, the same size as the fragment obtained for the parental strain using the same primers. Two bands (of approximately 1600 and 1800 bp) were obtained for samples 2, 3, 8, 9, 12 (putative single mutants) and 14 to 19 (putative double mutants). Samples 21, 22 and 24 did not show any band.

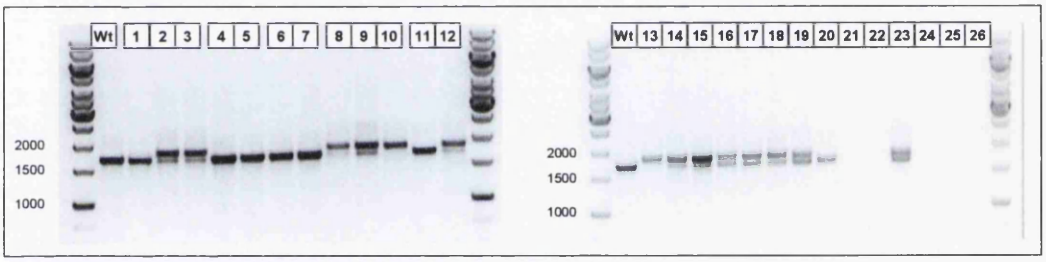


**Figure 18.** 0.8% agarose gels showing the PCR amplification of genomic DNA from the putative mutants using the primers 158A1conF1 and 158A1conR1 (flanking primers for the gene *sco6998* (CYP158A1)). First and last lane in each gel: 1 kb marker. Second lane: wild type DNA. The gel on the left is showing DNA from putative single mutants for the gene *sco6998* encoding for CYP158A1 (samples 1 to 12). The one on the right is showing DNA from putative double mutants (with gene for CYP158A2 previously deleted) (samples 13 to 26). The band in the "wt" lane is approximately 1600 bp. The second band in some of the lanes is approximately 1800 bp.

A band of approximately 1400 bp was obtained for samples 1, 2, 3, 8, 9, 10, 12 (putative single mutants for CYP158A1 genes) and 13, 14, 16, 18 to 21 (putative double mutants for CYP158A1 and CYP158A2 genes) when amplifying the DNA with the flanking primers for the cassette (1327 bp long) (figure 19).

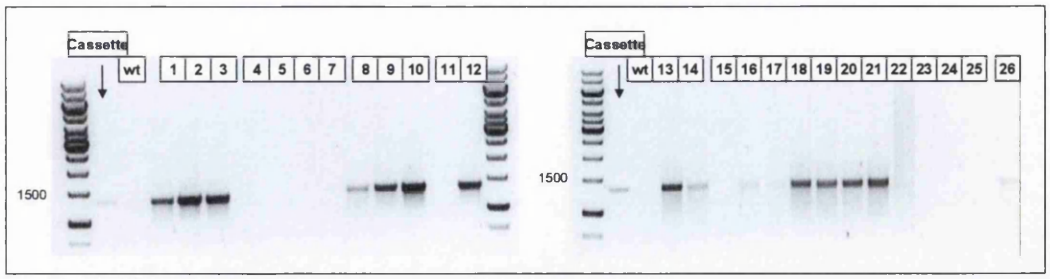


**Figure 19**. 0.8% agarose gels showing the PCR amplification of genomic DNA from the putative mutants using primers SpmCssF and SpmCssR, annealing at both ends of the spectinomycin cassette. First and last lane: 1 kb marker. Second lane: Spectinomycin cassette as probe. Third lane: wild type DNA. The gel on the left is showing DNA from putative single mutants for the gene *sco6998* encoding for CYP158A1 (samples 1 to 12). The one on the right is showing DNA from putative double mutants (with gene for CYP158A2 previously deleted) (samples 13 to 26). The size of the bands are 1400 bp approximately.

There was no amplification product detected for samples 4 to 7 of the putative single mutants for CYP158A1 gene, and 24 to 26 of the putative double mutants for CYP158A1 and CYP158A2 genes when amplifying DNA with a primer annealing inside the cassette, like for the parental strain. The rest of samples showed an amplification product of approximately 800 bp (figure 20).

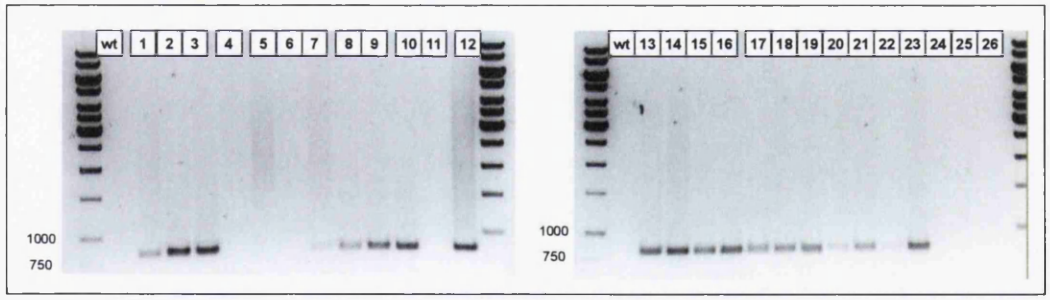


**Figure 20.** 0.8% agarose gels showing the PCR amplification of genomic DNA from the putative mutants using primers SpecIntF (annealing inside the spectinomycin cassette) and 158A1conR1 (reverse flanking primer for the gene *sco6998*). First and last lane: 1 kb marker. Second lane: wild type DNA. The gel on the left is showing DNA from putative single mutants for the gene *sco6998* encoding for CYP158A1 (samples 1 to 12). The one on the right is showing DNA from putative double mutants (with gene for CYP158A2 already deleted) (samples 13 to 26). The size of the bands is approximately 800 bp.

### 3.1.4 Confirmation by Sequencing

In addition to PCR screening we performed DNA sequencing of the diagnostic fragments in order to confirm the gene deletion event. Primers flanking

the locus of the gene *sco6998* at both ends were used for sequencing in order to detect the insertion of the cassette in the chromosome by comparing the sequence of the junction with the sequence of the parental strain.

PCR reactions (figure 21) were carried out with samples 3,10 (putative single mutants), 13 and 21 (putative double mutants) in order to amplify a DNA fragment corresponding to the *sco6998* locus (with primers 158A1conF1 and 158A1conR1, see annexe 2.) and another fragment containing only part of the spectinomycin cassette (primers SpecIntF annealing inside the spectinomycin resistance gene and 158A1conR1).

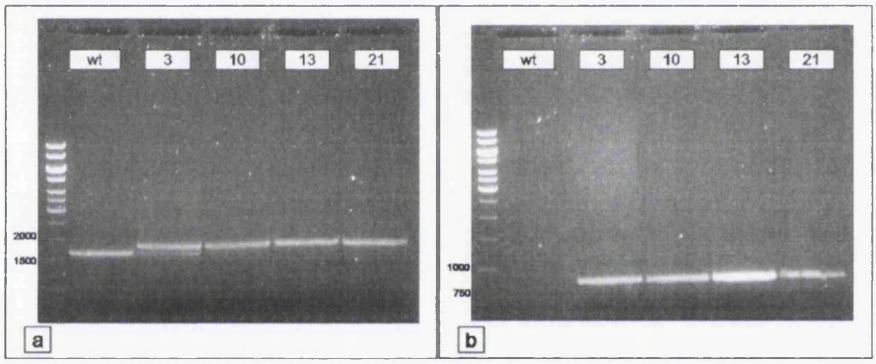


**Figure 21. a)** PCR with flanking primers 158A1conF1 and 158A1conR1 for the gene *sco6998*. Samples 3 and 10: putative single mutants. Samples 13 and 21: putative double mutants. The bands obtained are approximately 1600 bp for the wild type and the smaller band of the sample 3 and approximately 1800 bp for the rest of the bands. **b)** PCR with SpecIntF, annealing inside the spectinomycin cassette, and 158A1conR1. The bands obtained are approximately 800 bp.

All the bands that appeared after running the samples in agarose gels were extracted and sent for sequencing using appropriate primers. The sequence obtained for every sample was aligned and compared with the sequence of the chromosomal DNA contained in the cosmid 8F11 and the sequence of the spectinomycin cassette contained in the plasmid pIJ778 (both sequences were found in the GenBank® database). All the alignments were carried out with the pair-wise alignment tool available in the software Vector NTI 9.0 (Informax).

Primers 158A1conF1 and 158A1conR1 (annealing 225 and 202 bp respectively from each end of the gene *sco6998*, figure 22) were used for sequencing of both sides of the gene *sco6998* locus in order to try to confirm the replacement of

the gene by the spectinomycin cassette. The position and orientation of the gene within the chromosome is shown in figure 23. Within the cosmid, the gene *sco6998* is located between bases 25688 and 26911.

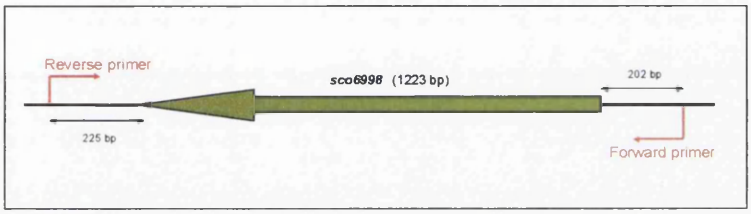


**Figure 22.** Position of the *sco6998* flanking primers 158A1conF1 (forward) and 158A1conR1 (reverse).

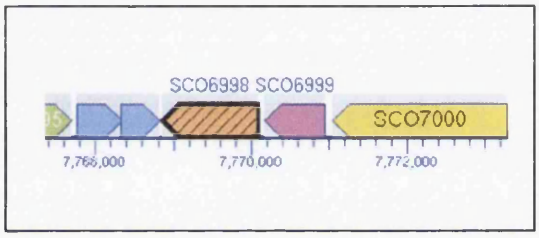


**Figure 23.** Position of the gene *sco6998* with respect to the chromosome. Image extracted from ScoCyc (http://scocyc.streptomyces.org.uk:14980/SCO/NEW-IMAGE?type=FULL-MAP&object=SCO6998)

SpecIntF (a primer annealing inside the spectinomycin resistance gene, see figure 24) was also used to sequence as an additional proof of the insertion of the spectinomycin cassette.

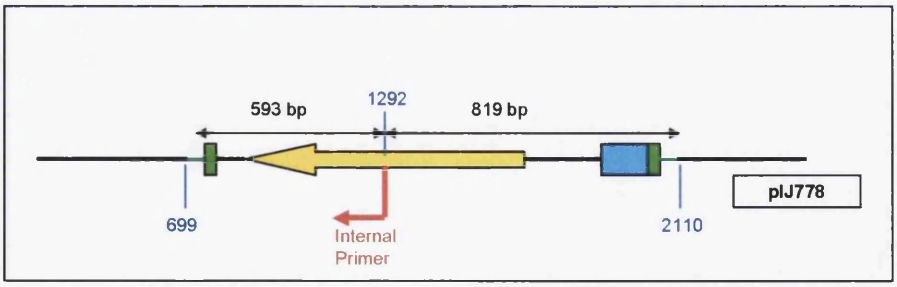


**Figure 24.** Position of the internal primer (SpecIntF) used for sequencing and the spectinomycin cassette with respect to the plasmid pIJ778. The location in the plasmid is indicated in blue. Spectinomycin cassette is located between positions 699 and 2110 within the plasmid. The internal primer anneals in position 1292 of the plasmid, 593 bp away from one of its ends.

The DNA sequence obtained from the parental strain DNA (figure 25), used as a control, coincides almost completely with the cosmid 8F11 sequence in both

sides of the *sco6998* locus. There is no coincidence with the plasmid pIJ778 sequence in either side.

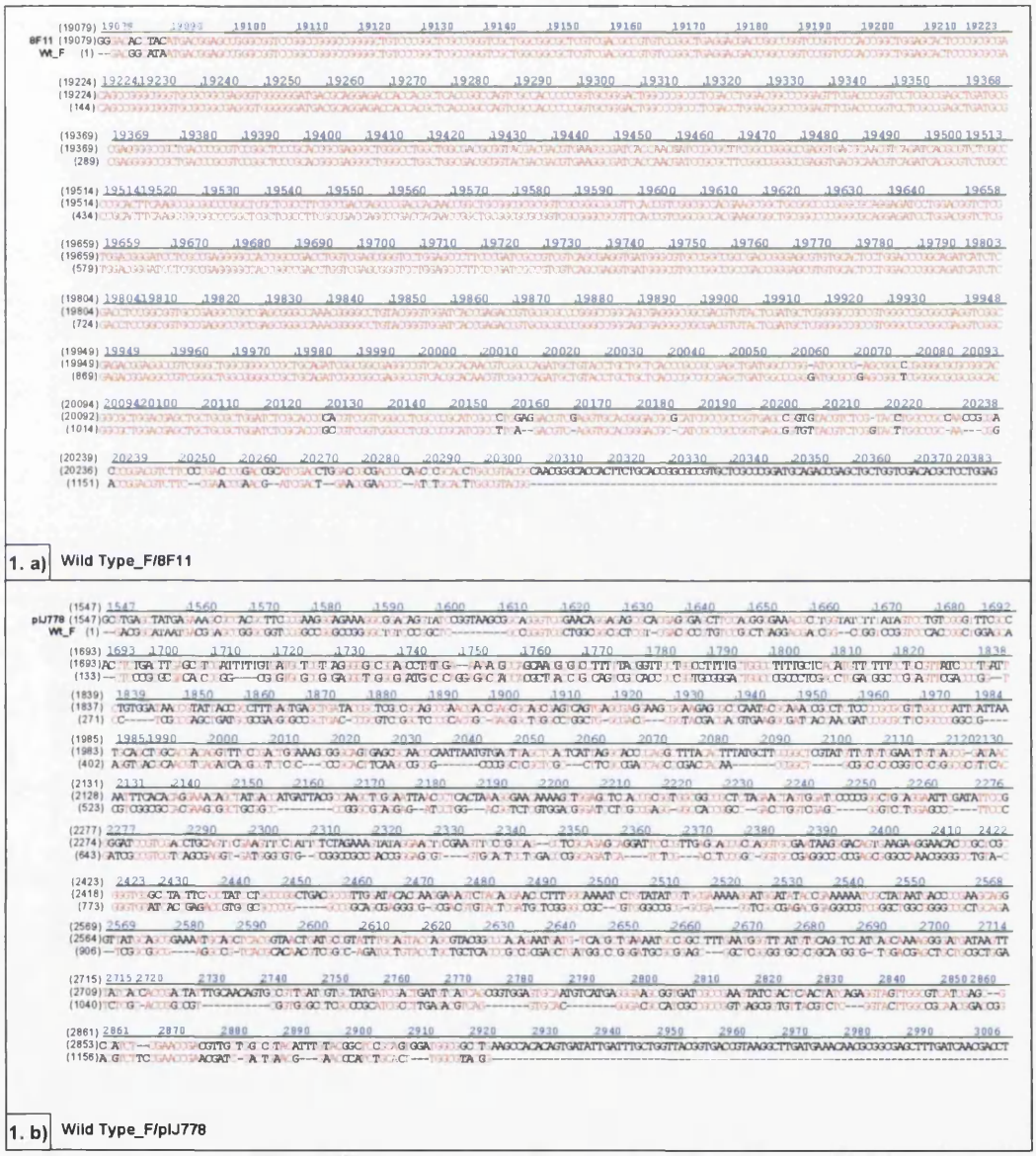


**Figure 25.1** Control DNA (Wild type) sequence from the right side of the *sco6998* locus (using primer 158A1conF1) aligned with the cosmid 8F11 (a) and the spectinomycin cassette (b) sequence. The coinciding bases are shown in red.

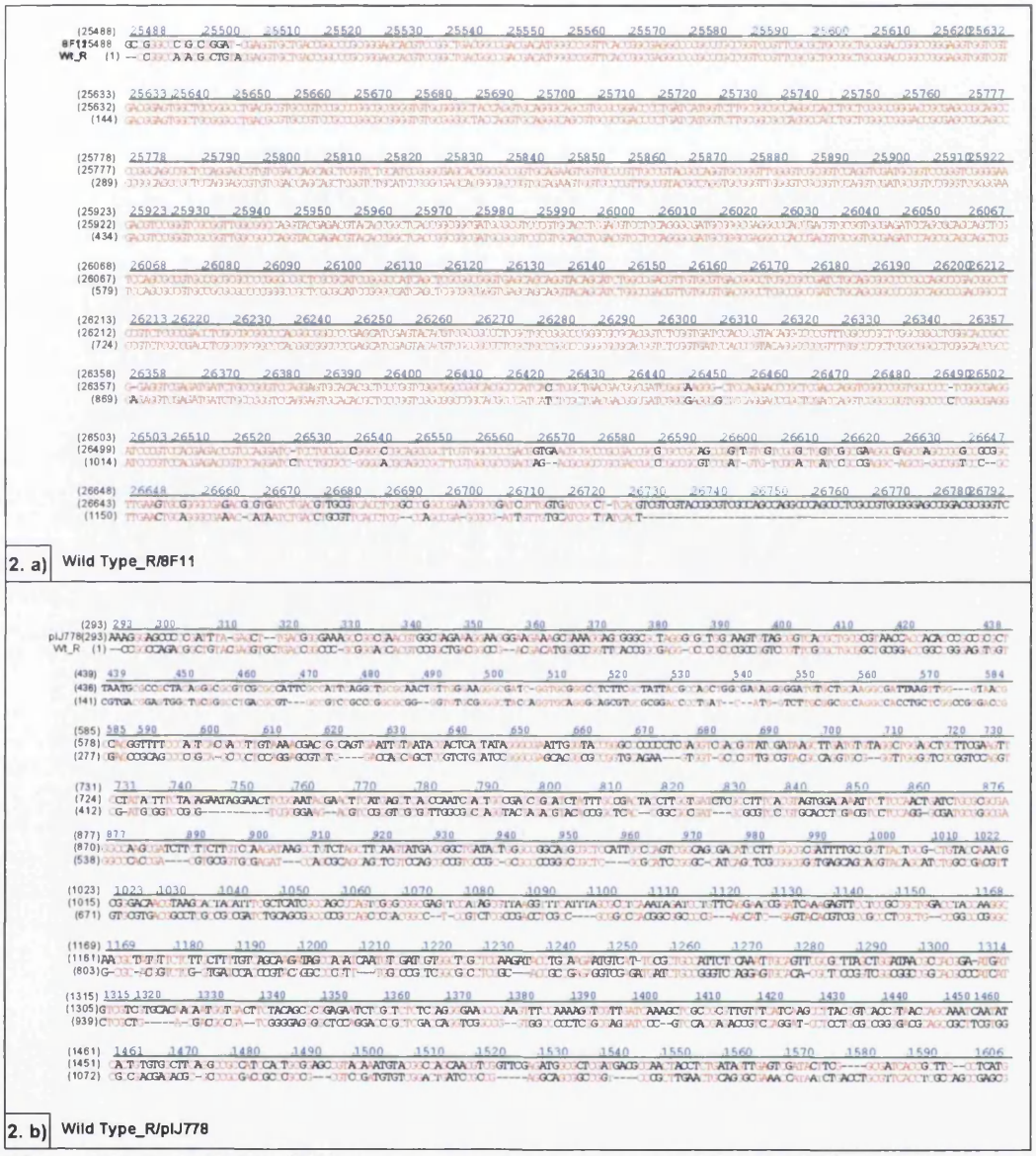


**Figure 25.2** Control DNA (Wild type) sequence from the left side of the *sco6998* locus (using primer 158A1conR1) aligned with the cosmid 8F11 (a) and the spectinomycin cassette (b) sequence. The coinciding bases are shown in red.

Two bands were obtained for sample 3 (putative single mutant) when amplifying the fragment corresponding to the *sco6998* locus. The DNA sequence obtained for the largest band (figure 26) coincides with the parental chromosomal sequence only in the regions flanking the *sco6998* reading frame. The DNA sequence which would correspond to the *sco6998* internal coding sequence in this sample shows coincidence with the sequence of the spectinomycin cassette.

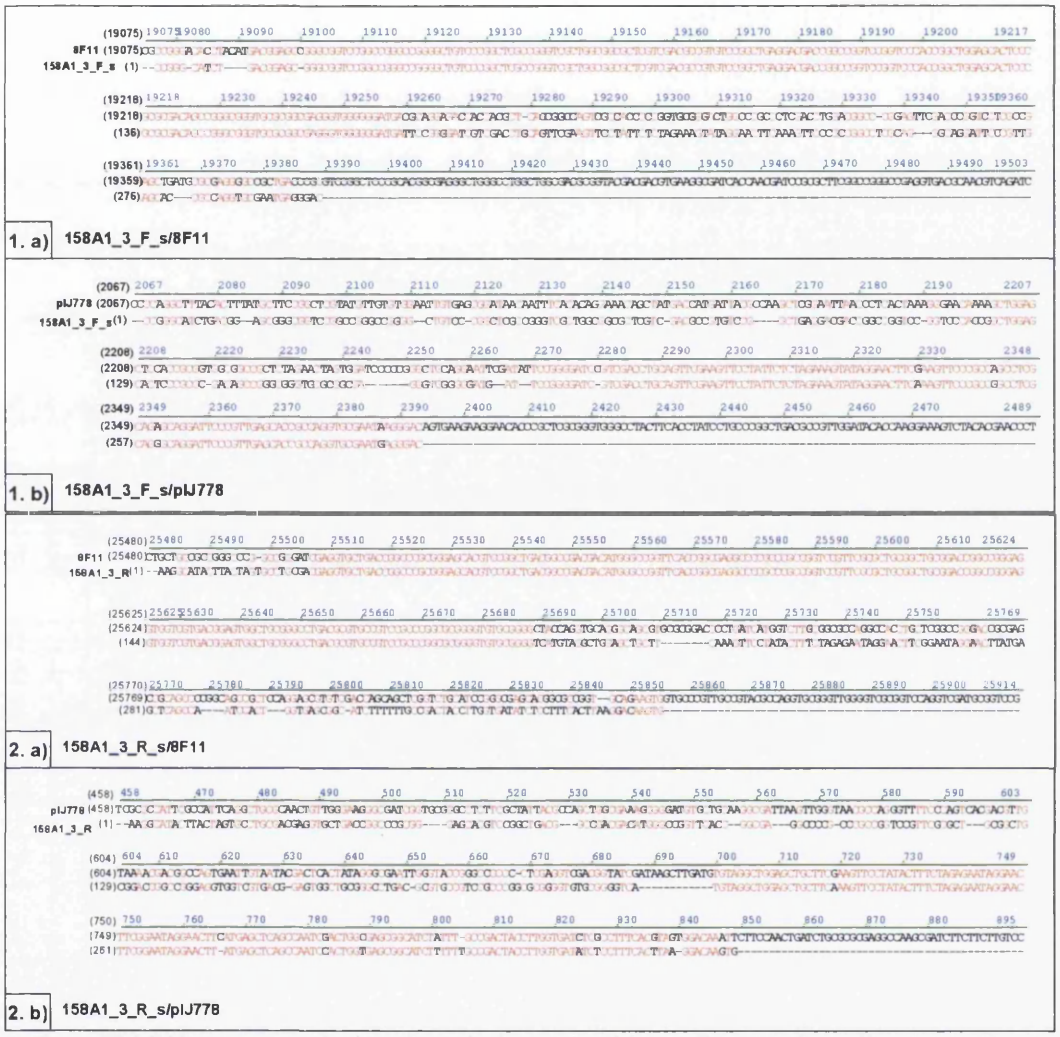


**Figure 26.** DNA sequence obtained from the largest band in sample 3 (see figure 21. a) (putative single mutant for CYP158A1 gene), from the right side (using forward primer 158A1conF1) (1) and from the left side of the gene (using reverse primer 158A1conR1) (2), aligned with the chromosomal DNA sequence contained in the cosmid 8F11 sequence (a) and with the spectinomycin cassette sequence contained in plasmid pIJ778(b). The coinciding bases are shown in red.

The DNA sequence obtained from the smallest band for sample 3 (figure 27) shows only coincidence with the chromosomal sequence and not with the spectinomycin cassette.

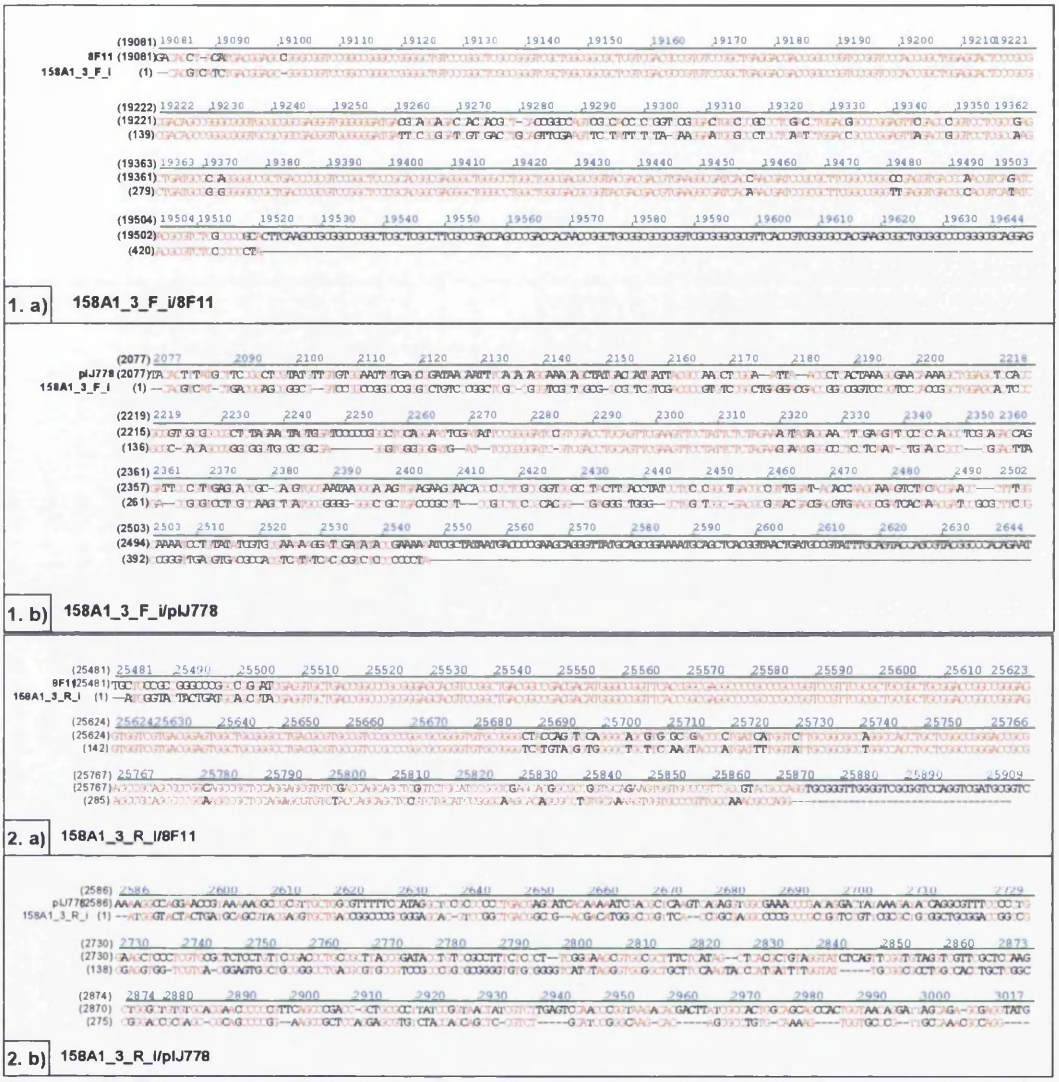


**Figure 27.** DNA sequence obtained from the smallest band in sample 3 (figure 21. a) (putative single mutant for CYP158A1 gene), from the right side (using forward primer 158A1conF1) (1) and from the left side of the gene (using the reverse primer 158A1conR1) (2), aligned with the chromosomal DNA sequence contained in the cosmid 8F11 sequence (a) and with the spectinomycin cassette sequence contained in pIJ778 (b). The coinciding bases are shown in red.

The DNA sequence obtained from samples 10 (putative single mutant) (figure 28), 13 and 21 (putative double mutants) (figures 29 and 30 respectively) coincides with the chromosomal sequence only in the region flanking the *sco6998* reading frame. The sequence which would correspond to the *sco6998* internal coding sequence coincides with the spectinomycin cassette sequence.

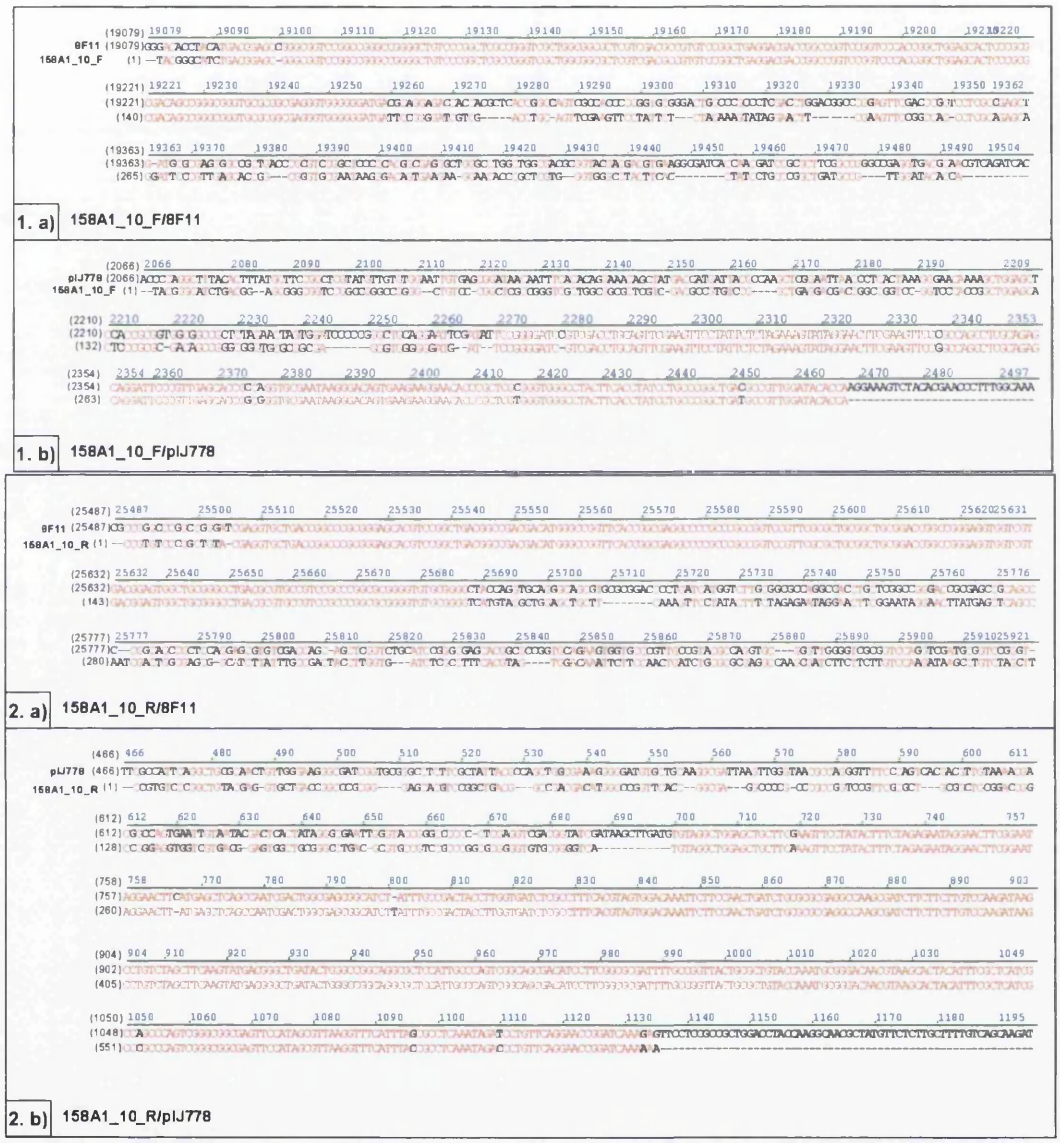


**Figure 28.** DNA sequence obtained from sample 10 (figure 21. a) (putative single mutant for CYP158A1 gene), from the right side (using forward primer 158A1conF1) (1) and from the left side of the gene (using the reverse primer 158A1conR1) (2), aligned with the chromosomal DNA sequence contained in the cosmid 8F11 sequence (a) and with the spectinomycin cassette sequence contained in pIJ778 (b). The coinciding bases are shown in red.

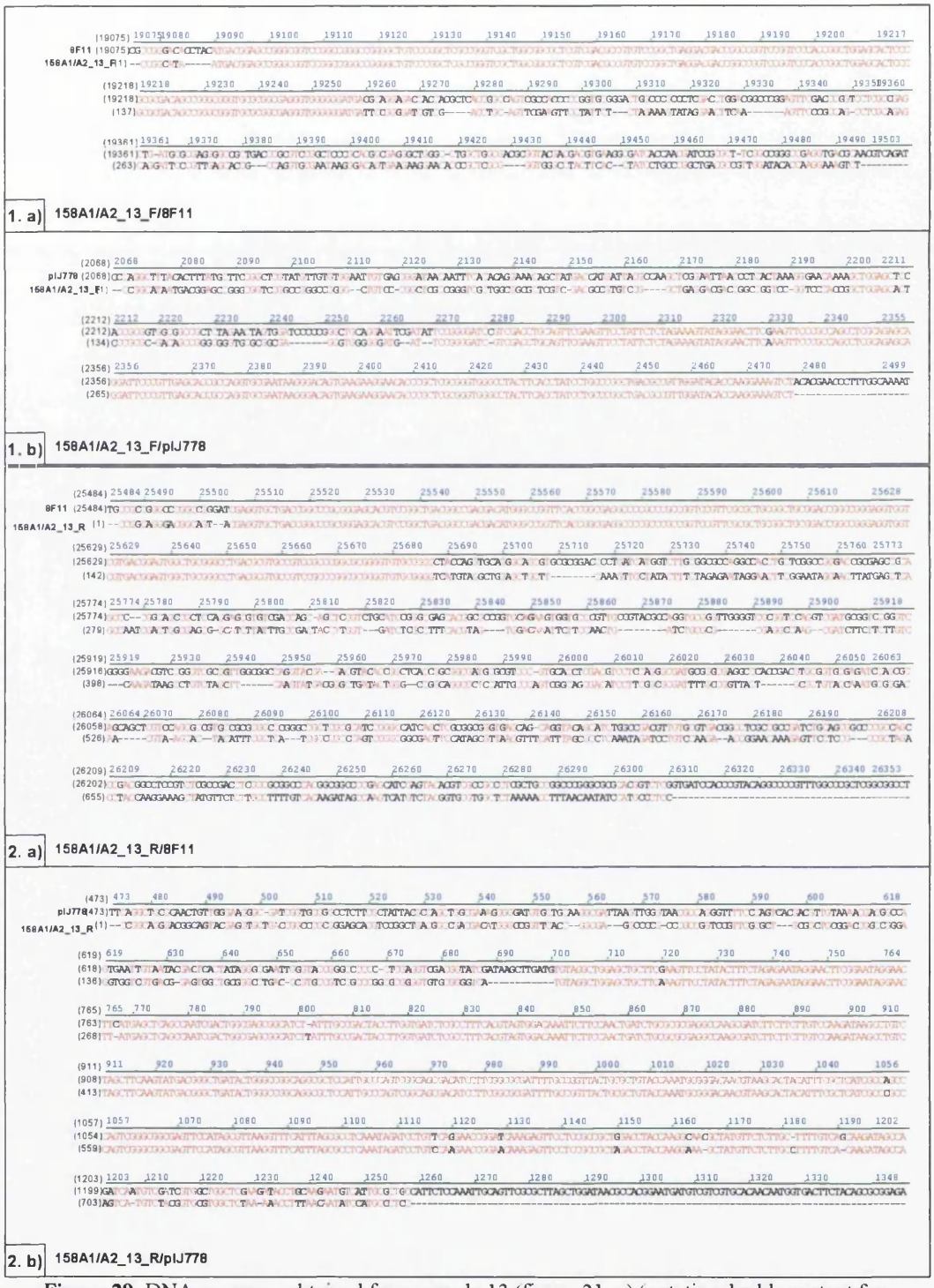


**Figure 29.** DNA sequence obtained from sample 13 (figure 21. a) (putative double mutant for CYP158A1 gene), from the right side (using forward primer 158A1conF1) (1) and from the left side of the gene (using the reverse primer 158A1conR1) (2), aligned with the chromosomal DNA sequence contained in the cosmid 8F11 sequence (a) and with the spectinomycin cassette sequence contained in pIJ778 (b). The coinciding bases are shown in red.

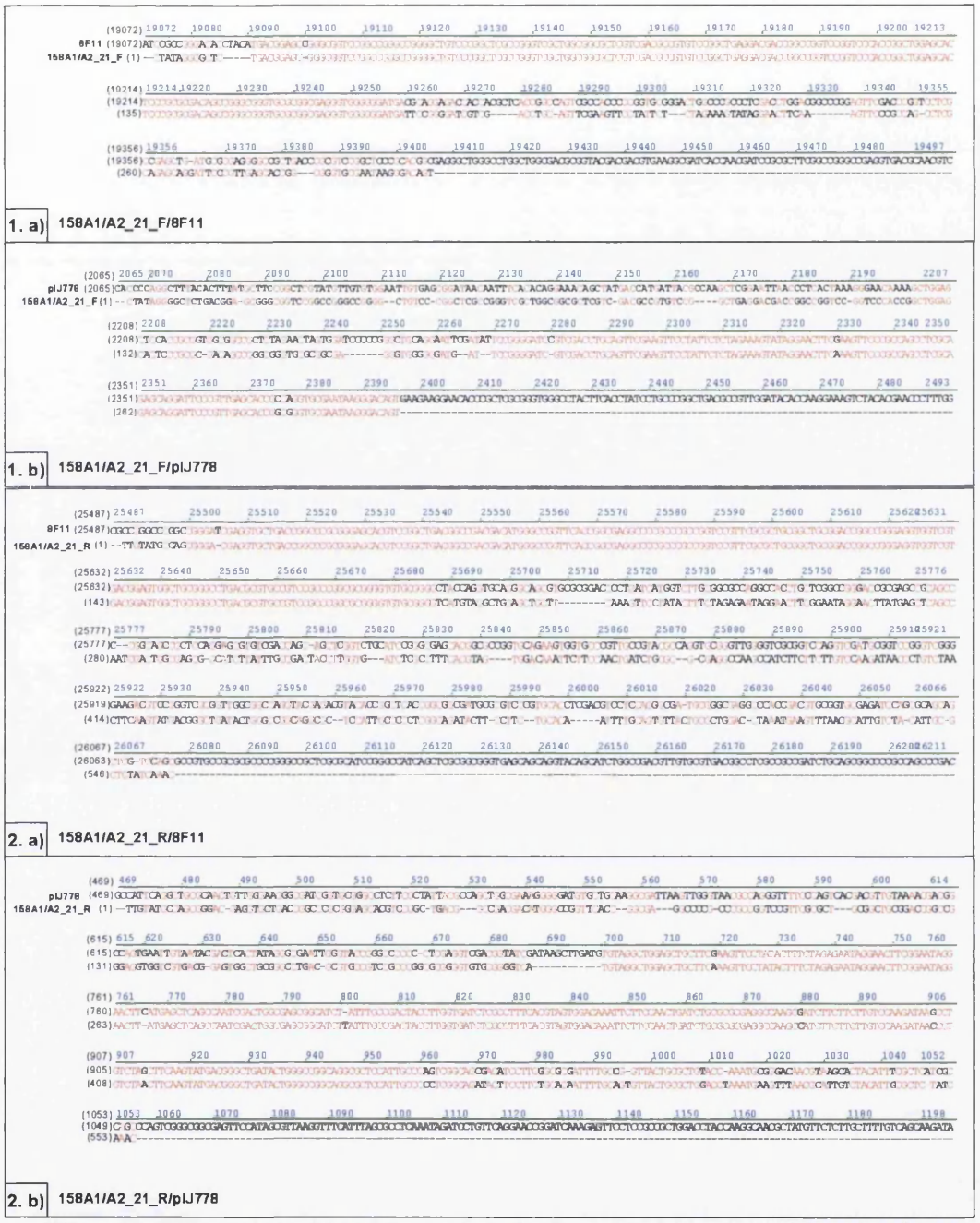


**Figure 30.** DNA sequence obtained from sample 21 (figure 21. a) (putative double mutant for CYP158A1 gene), from the right side (using forward primer 158A1conF1) (1) and from the left side of the gene (using the reverse primer 158A1conR1) (2), aligned with the chromosomal DNA sequence contained in the cosmid 8F11 sequence (a) and with the spectinomycin cassette sequence contained in pIJ778 (b). The coinciding bases are shown in red.

The sequence obtained from the DNA fragments amplified by primer SpecIntF (annealing inside the spectinomycin gene) and 158A1conR1 (figure 21. b) was also compared to the chromosomal sequence contained in the cosmid 8F11 and the spectinomycin cassette sequence contained in the plasmid pIJ778 (figures 31 to 34). There is coincidence between the sequence of the samples and the sequence of

the spectinomycin cassette up to the position where the *sco6998* coding sequence ends.

The sequence obtained from the left side of the fragments obtained coincides with the chromosomal sequence up to the end of the *sco6998* reading frame. From that position these sequences coincide with the sequence of the spectinomycin cassette.

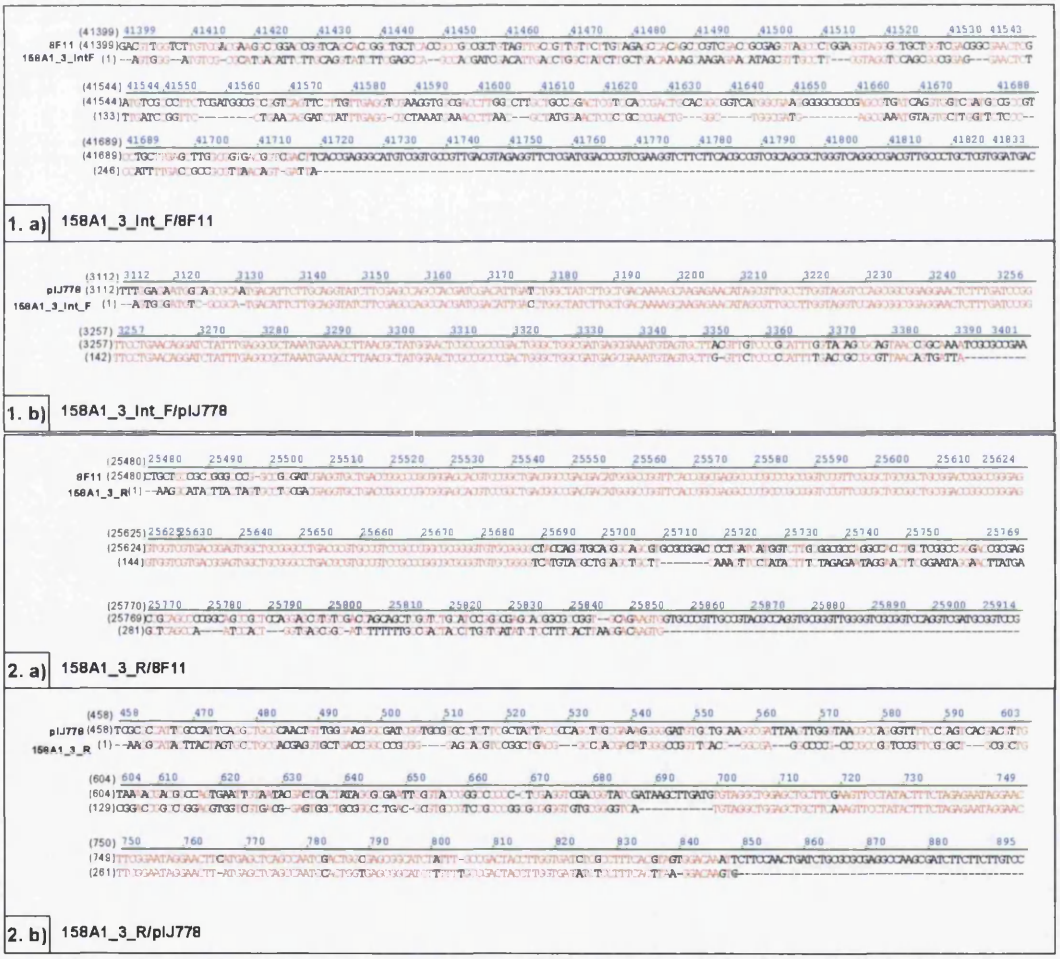


**Figure 31.** DNA sequence obtained from sample 3 (figure 21. b) (putative single mutant for CYP158A1 gene), from the right side (using primer SpecIntF) (1) and from the left side of the amplified fragment obtained (using the reverse primer 158A1conR1) (2), aligned with the chromosomal DNA sequence contained in the cosmid 8F11 sequence (a) and with the spectinomycin cassette sequence contained in pIJ778 (b). The coinciding bases are shown in red.


**Figure 32.** DNA sequence obtained from sample 10 (figure 21. b) (putative single mutant for CYP158A1 gene), from the right side (using primer SpecIntF) (1) and from the left side of the amplified fragment obtained (using the reverse primer 158A1conR1) (2), aligned with the chromosomal DNA sequence contained in the cosmid 8F11 sequence (a) and with the spectinomycin cassette sequence contained in pIJ778 (b). The coinciding bases are shown in red.

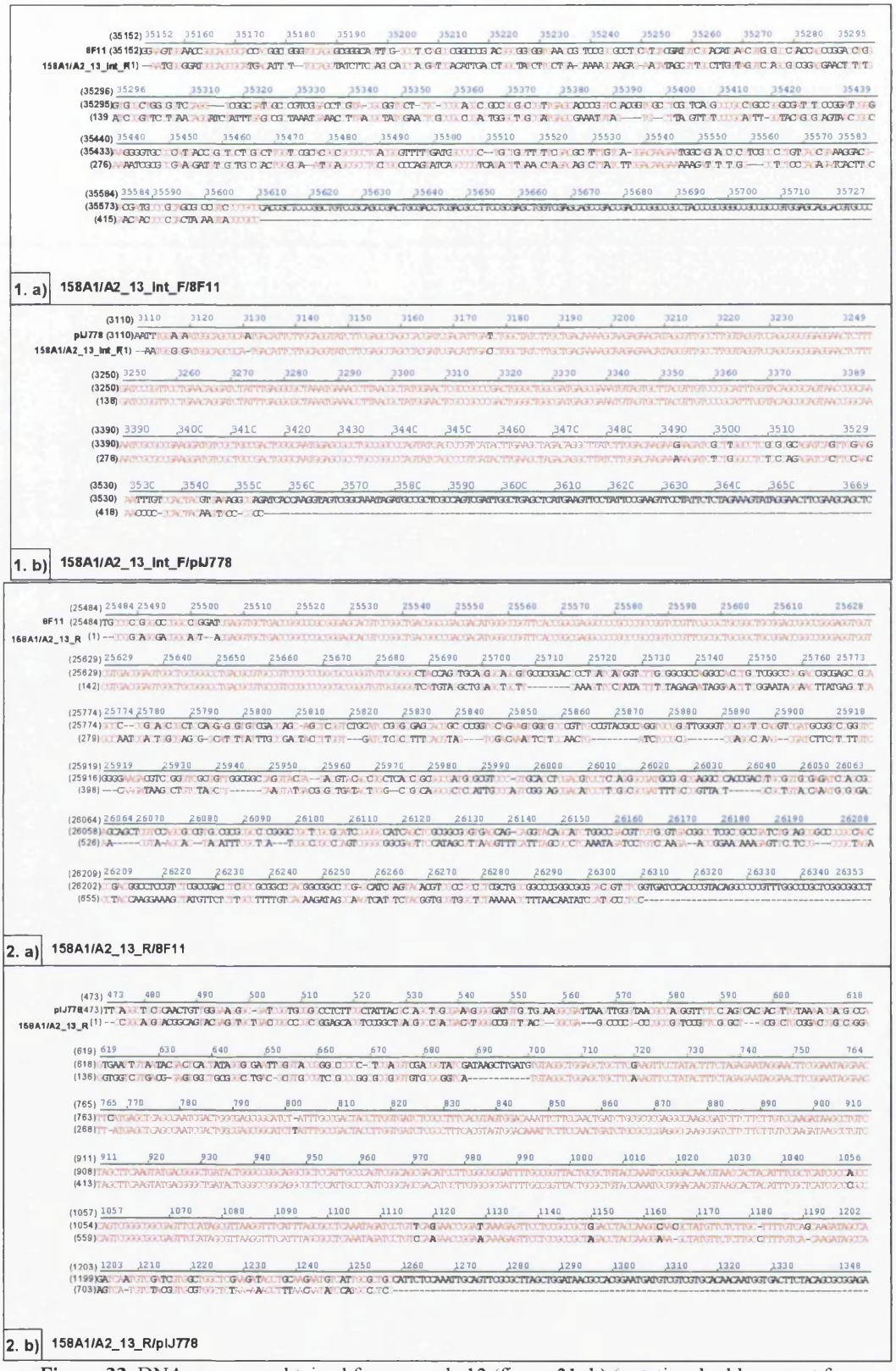


**Figure 33.** DNA sequence obtained from sample 13 (figure 21. b) (putative double mutant for CYP158A1 gene), from the right side (using primer SpecIntF) (1) and from the left side of the amplified fragment obtained (using the reverse primer 158A1conR1) (2), aligned with the chromosomal DNA sequence contained in the cosmid 8F11 sequence (a) and with the spectinomycin cassette sequence contained in pIJ778 (b). The coinciding bases are shown in red.

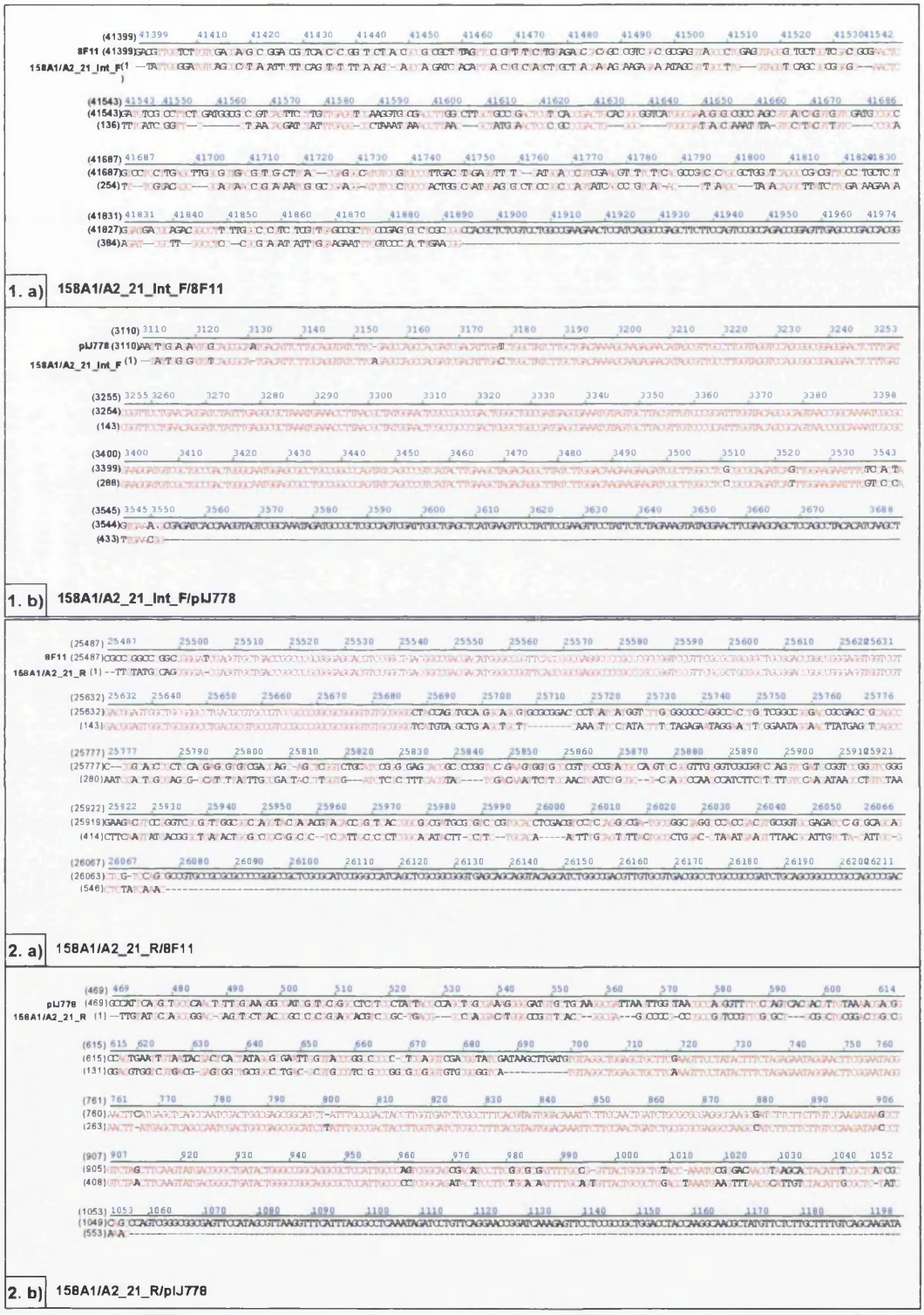


**Figure 34.** DNA sequence obtained from sample 21 (figure 21. b) (putative double mutant for CYP158A1 gene), from the right side (using primer SpecIntF) (1) and from the left side of the amplified fragment obtained (using the reverse primer 158A1conR1) (2), aligned with the chromosomal DNA sequence contained in the cosmid 8F11 sequence (a) and with the spectinomycin cassette sequence contained in pIJ778 (b). The coinciding bases are shown in red.

These results show the sequence of the junction between the spectinomycin cassette and the adjacent region of the *sco6998* locus in the mutants, showing coincidence with the parental chromosomal sequence at the flanks of the locus, and with the spectinomycin cassette inside the coding region. Only the sequence from the smaller band for sample 3 showed coincidence with the chromosomal sequence all along its length. A single cross-over is likely to have occurred during recombination for this mutant and the gene deletion was unsuccessful. Further corroboration by Southern blotting is required for validation of mutations.

## 3.2 STUDIES ON PHENOTYPE OF THE MUTANTS COMPARED TO THE PARENTAL STRAIN

Spores of the parental strain, the putative single deletion mutants for the CYP158A1 gene, and the putative double mutants for both CYP158A1 and CYP158A2 genes were inoculated in liquid and solid media in order to detect any differences on phenotype. These studies were in parallel with the mutant verification studies. Samples of isolate 3 were included in this analysis although the deletion of the gene *sco6998* was unsuccessful as indicated by PCR analysis and DNA sequencing. Further phenotype studies reported below were undertaken using the mutant strains as described although more validation is still needed.

- **Cultures in liquid medium (YEME):**

After 7 days of incubation cultures in YEME medium were observed to detect any changes in phenotype. The cultures were checked everyday for any appearance of colour in the medium.

No differences on phenotype with respect to the wild type strain were found in any of the samples (figures 35 to 37).

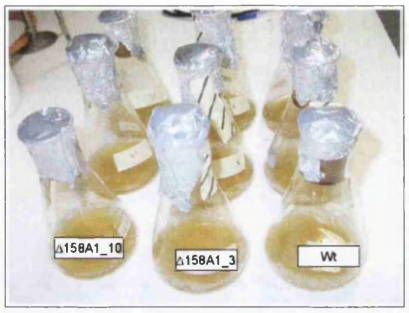


**Figure 35.** YEME cultures of wild type and putative single mutants for CYP158A1 after 7 days of incubation.

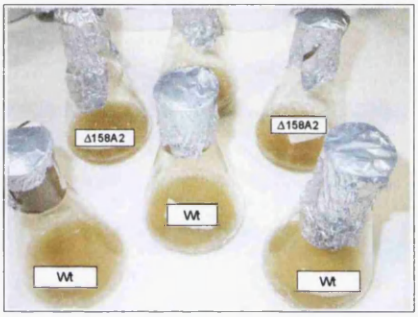


**Figure 36.** YEME cultures of wild type and mutants for CYP158A2 after 7 days of incubation.

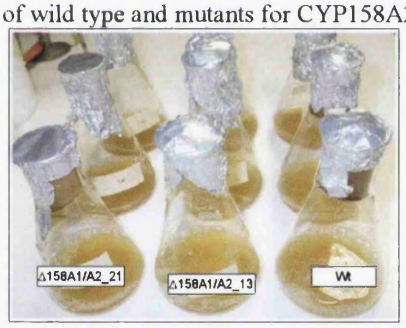


**Figure 37.** YEME cultures of wild type and double mutants after 7 days of incubation.

- **Cultures on solid medium (MS plates):**

An aliquote of spores was inoculated in MS medium plates which were incubated for a week. Four control plates were also set inoculating parental spores in MS medium plates with and without apramycin (50 μg/ml) given that the deletion of the CYP158A2 gene was performed by replacement with an apramycin resistance cassette and spectinomycin (400 μg/ml) because of the spectinomycin resistance cassette used to replace the CYP158A1 gene (figure 38).

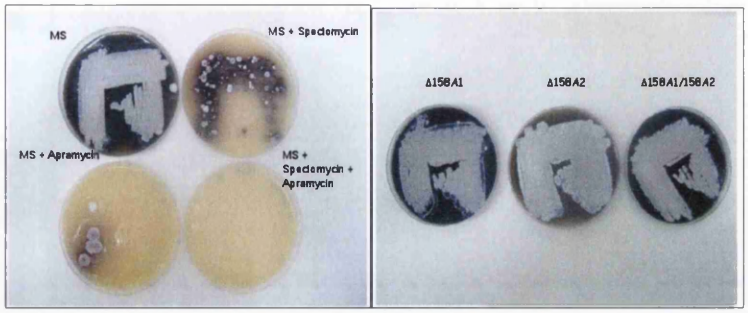


**Figure 38**. Spores of wild type and mutants inoculated in MS plates. a) Control plates. Wild type spores were inoculated in MS plates with and without antibiotics (CYP158A2 was disrupted with an apramycin resistance cassette). b) Spores of the putative single and double mutants for CYP158A1 and spores of the mutant for CYP158A2 inoculated in MS plates with their respective antibiotics.

No significant differences were detected in the phenotype of the single mutants for the CYP158A1 and CYP158A2 encoding genes or of the double mutant for both genes in comparison with the parental strain in solid medium.

According to these results, deletion of the gene *sco6998* encoding for CYP158A1, of the gene *sco1207* encoding for CYP158A2, or deletion of both genes in the same strain, does not seem to have any effect on the phenotype in the conditions tested.

## 3.3 UV SENSITIVITY ASSAYS

The results of the spore survival analysis after UV irradiation are shown in figure 39 and table 2. The percentage of spore survival was calculated as the ratio between the colonies that appeared after 2 days of incubation after UV irradiation and the colonies that appeared at time 0, without any UV treatment.

| Dose (sec) | wt | Δ158A1_10 | Δ158A2 | Δ158A1/A2_13 |
|---|---|---|---|---|
| **0** | 100% | 100% | 100% | 100% |
| **5** | 27 | 3.37 | 98.28 | 94.38 |
| **10** | 37.3 | 5.42 | 91.43 | 93.97 |
| **15** | 32.2 | 4.94 | 74.35 | 94.78 |
| **20** | 18,6 | 4.19 | 81.18 | 97.11 |
| **30** | 17.5 | 3.46 | 57.25 | 66.26 |
| **40** | 18.04 | 3.99 | 63.23 | 69.39 |
| **50** | 9.8 | 4.04 | 36.74 | 72.33 |
| **60** | 4.29 | 1.66 | 36.74 | 59.98 |

**Table 2.** Percentage of colonies that survived after UV irradiation compared to the colonies that appeared without irradiation.

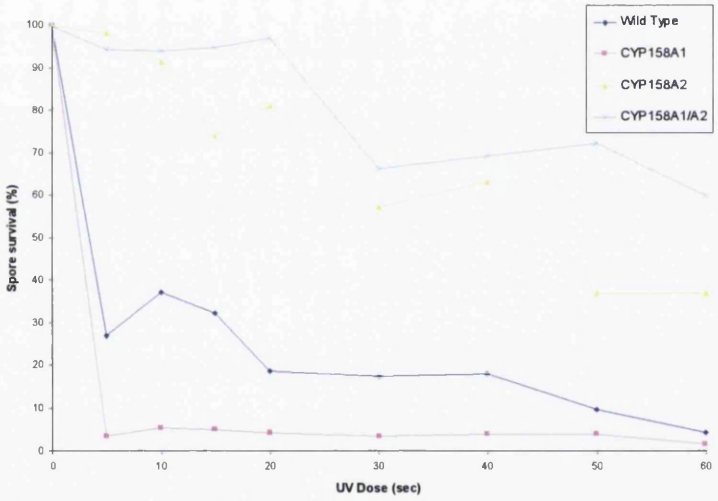


**Figure 39. Spore survival after UV irradiation.** Dark blue: parental strain. Pink: single mutant for CYP158A1 gene. Yellow: single mutant for CYP158A2 gene. Light Blue: double mutant for CYP158A1 and CYP158A2 genes.

## 3.4 MASS SPECTROMETRY

Liquid chromatography-coupled Mass spectrometry (HPLC/MS) was carried out to detect the presence of flaviolin in extracts of cultures inoculated with spores from the parental strain, the single mutant for the CYP158A1 gene, the single mutant for CYP158A2 gene and the double mutant for both genes. The spectra obtained are shown in figures 41 to 44.

Several compounds were initially separated by chromatography from each sample (showing as peaks in the chromatogram). These compounds were subjected to mass spectrometry and their mass spectra were extracted.

The mass spectra of the most relevant compounds detected (i.e the most abundant ones or the ones showing a peak with m/z value similar to that of flaviolin, figure 40) are shown in the figures below. The molecular mass of flaviolin is 206 amu.

FLAVIOLIN

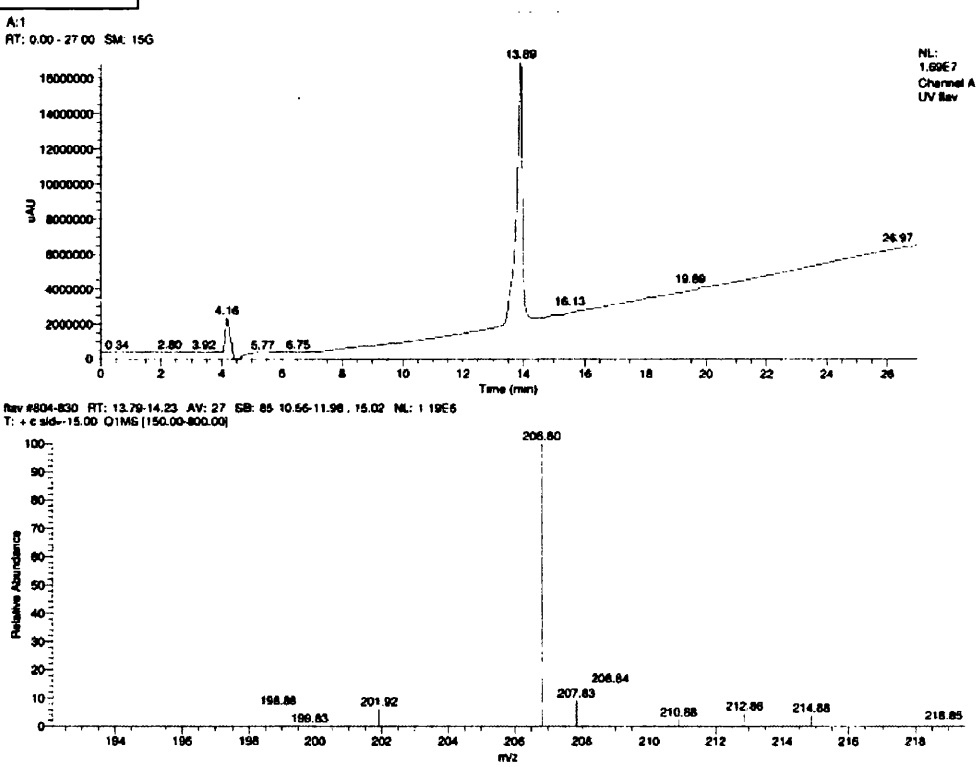


**Figure 40.** Flaviolin HPLC/MS spectra obtained in the same conditions as the samples. The top figure shows the total ion chromatogram (TIC) of the flaviolin eluting at 13.89 minutes. The figure at the bottom is the mass spectrum for this molecule which shows a base peak at an m/z value of 206.80

PARENTAL STRAIN

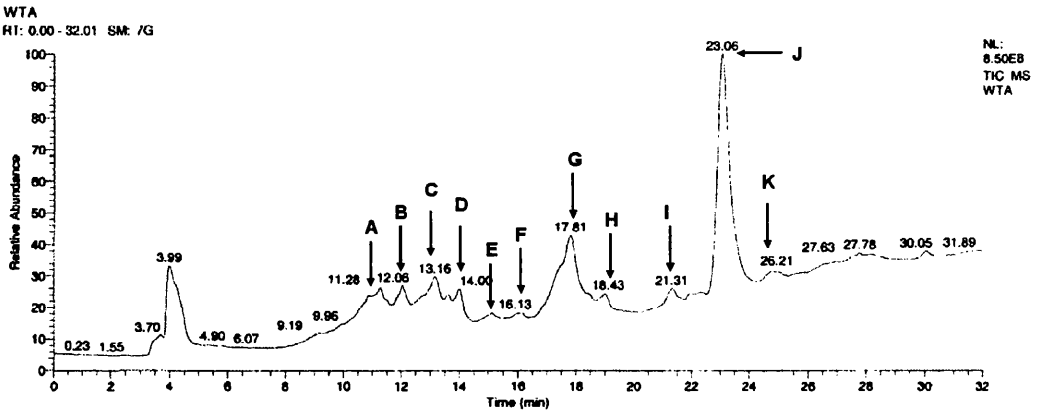


**Figure 41**. Total ion chromatogram (TIC) of the parental strain sample. Different compounds (peaks) were separated by HPLC eluting at different retention times. The figures 41.A to 41.K show the mass spectra of the peaks labelled in the chromatogram.

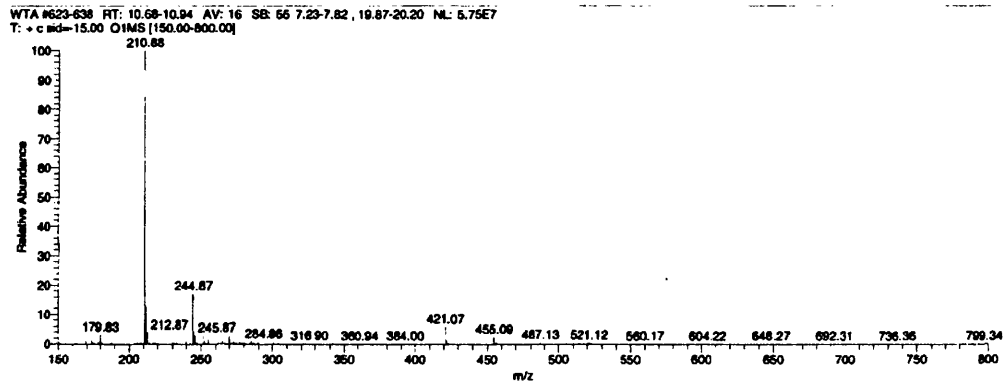


Figure 41.A: Mass spectrum of the compound eluting at 10.58 minutes.

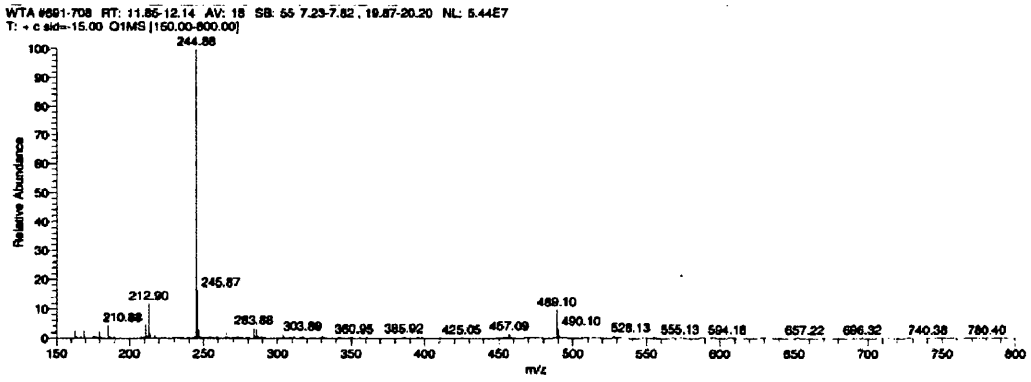


Figure 41.B: Mass spectrum of the compound eluting at 12.06 minutes.

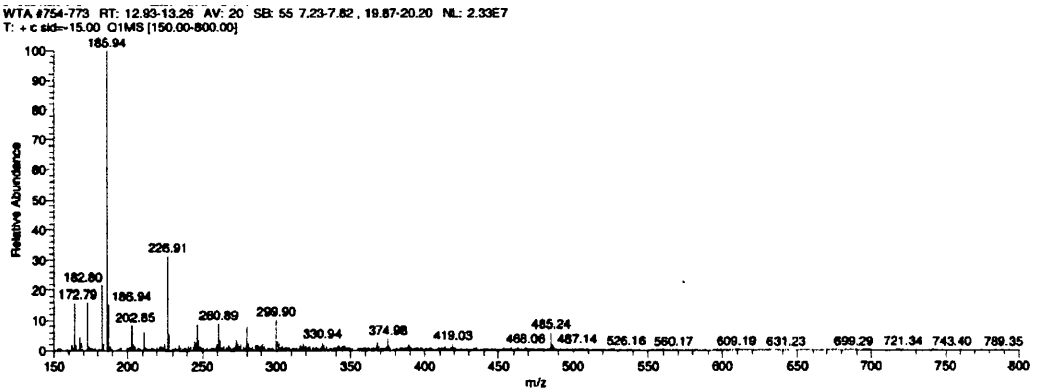


Figure 41.C: Mass spectrum of the compound eluting at 13.16 minutes

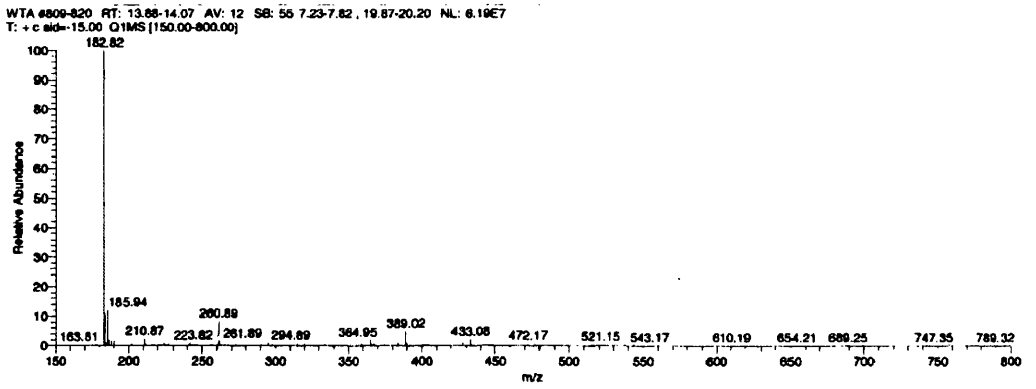


Figure 41.D: Mass spectrum of the compound eluting at 14 minutes.

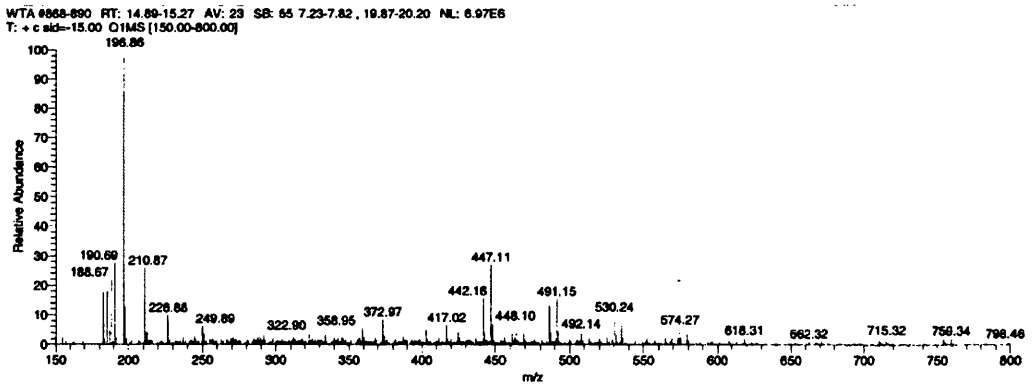


Figure 41.E: Mass spectrum of the compound eluting at 15 minutes.

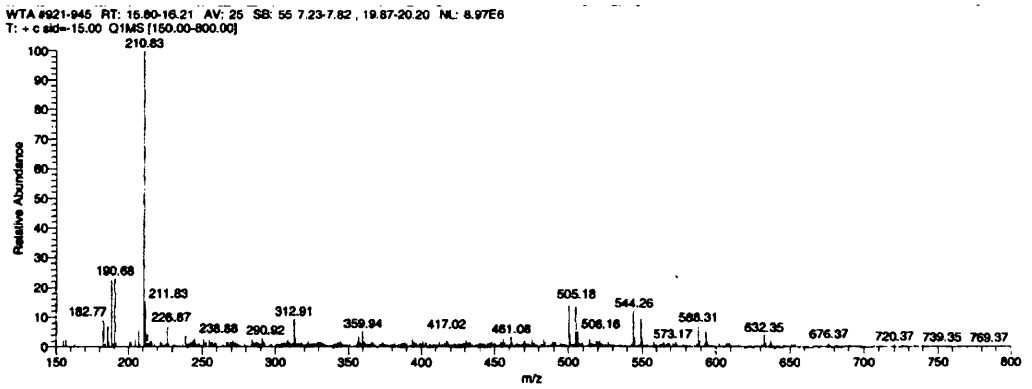


Figure 41.F: Mass spectrum of the compound eluting at 16.13 minutes.

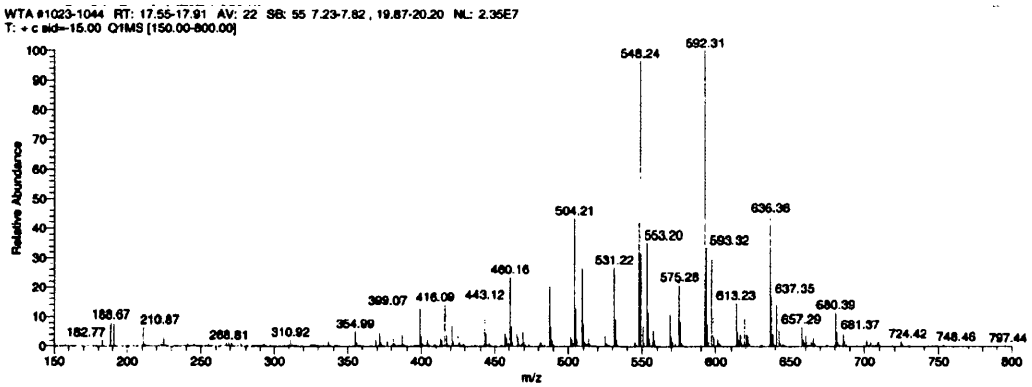


Figure 41.G: Mass spectrum of the compound eluting at 17.81 minutes.

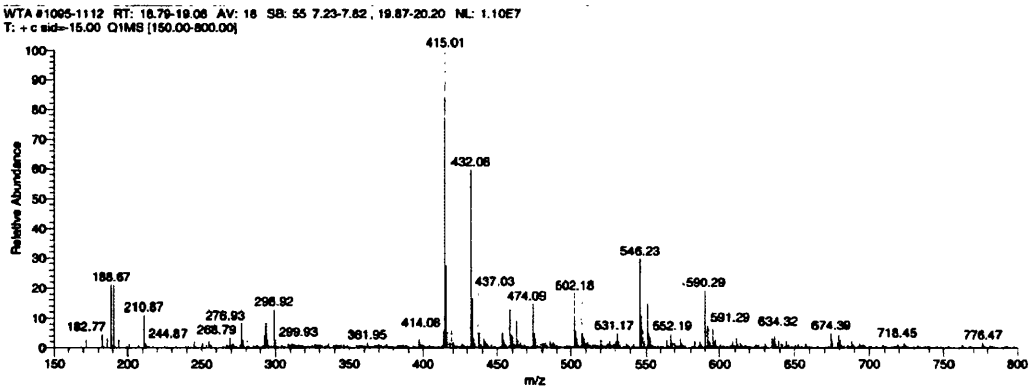


Figure 41.H: Mass spectrum of the compound eluting at 18.98 minutes.

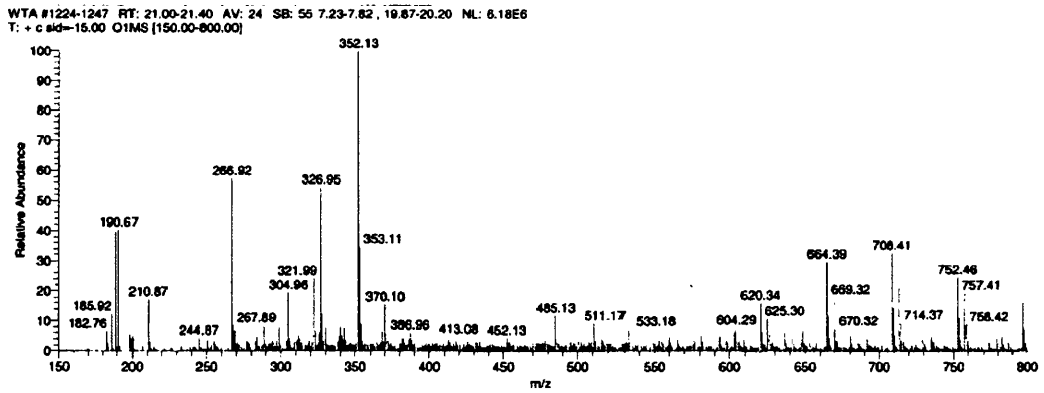


Figure 41.I: Mass spectrum of the compound eluting at 21.31 minutes.

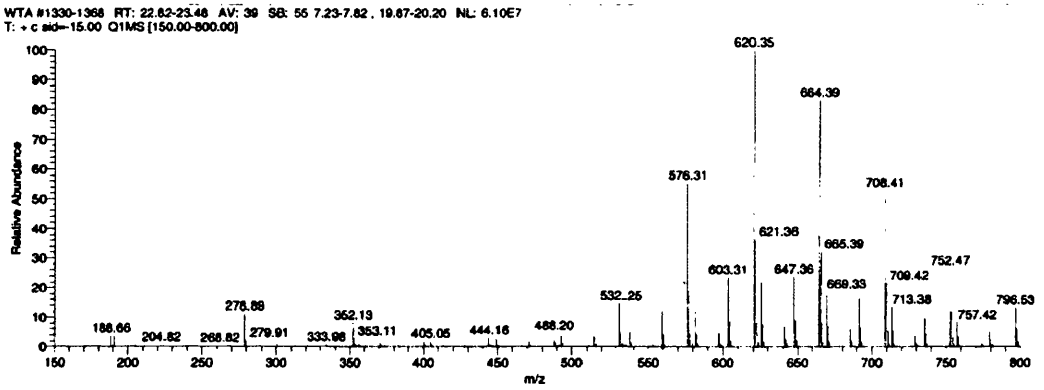


Figure 41.J: Mass spectrum of the compound eluting at 23.06 minutes.

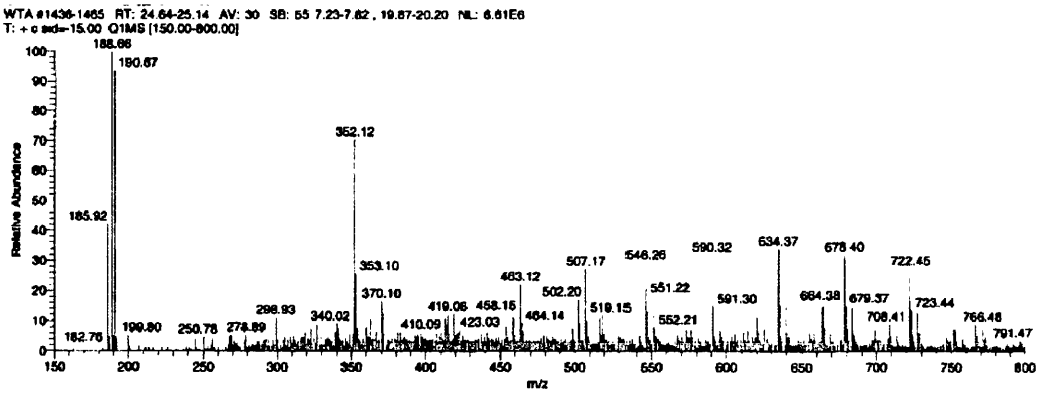


Figure 41.K: Mass spectrum of the compound eluting at 25 minutes.

ΔCYP158A1

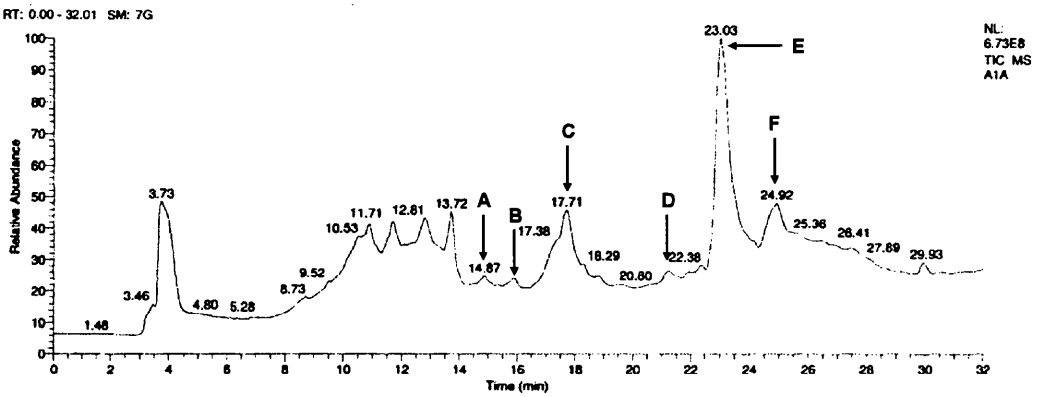


**Figure 42.** Total ion chromatogram (TIC) of the single mutant for CYP158A1 gene sample. The figures 42.A to 42.F show the mass spectra of the peaks labelled in the chromatogram.

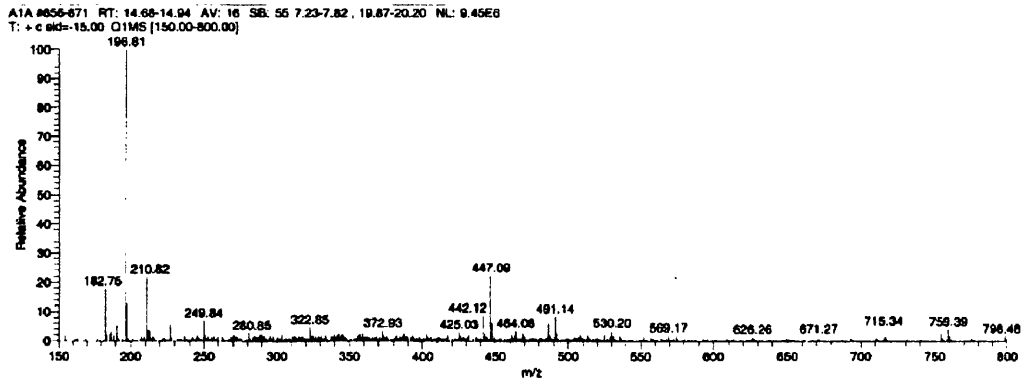


Figure 42.A: Mass spectrum of the compound eluting at 14.87 minutes.

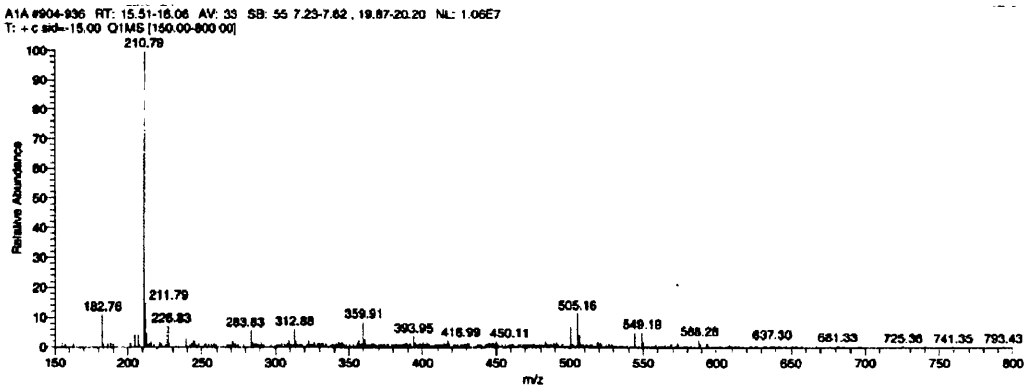


Figure 42.B: Mass spectrum of the compound eluting at 16 minutes.

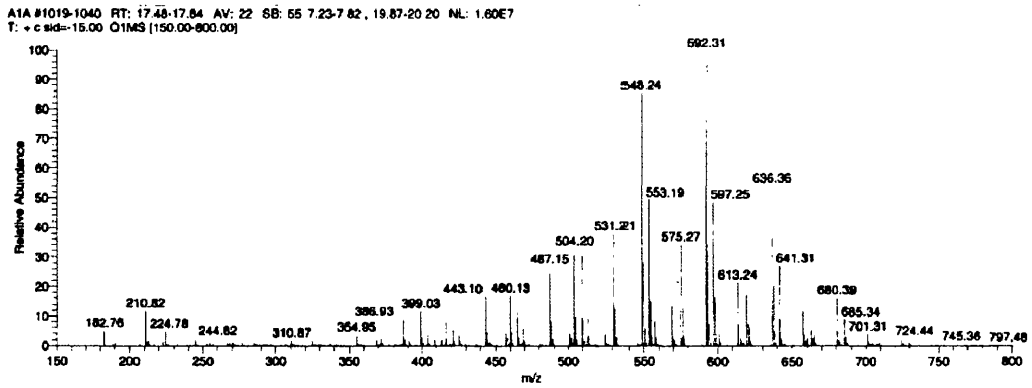


Figure 42.C: Mass spectrum of the compound eluting at 17.71 minutes.

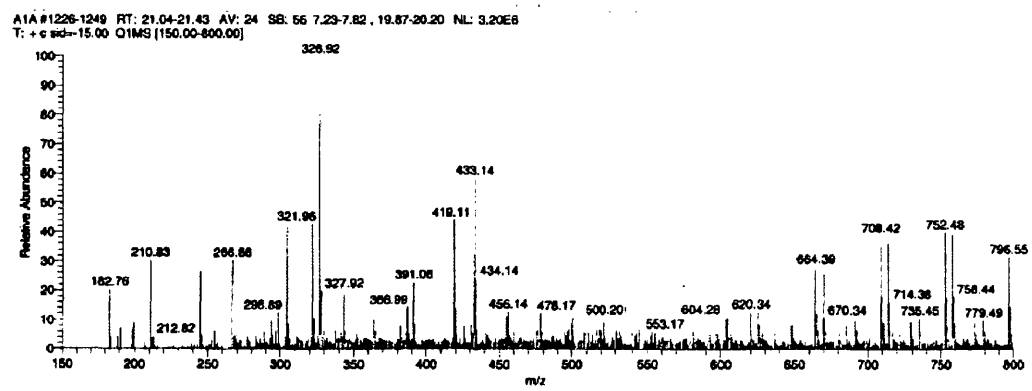


Figure 42.D: Mass spectrum of the compound eluting at 21.4 minutes.

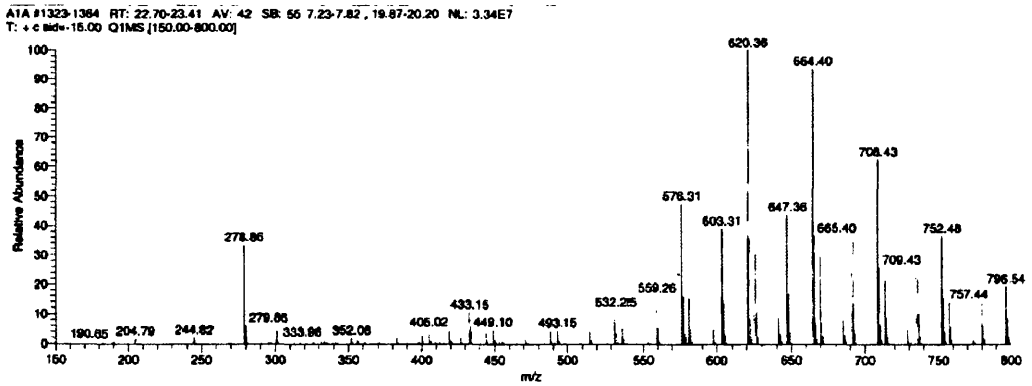


Figure 42.E: Mass spectrum of the compound eluting at 23.03 minutes.

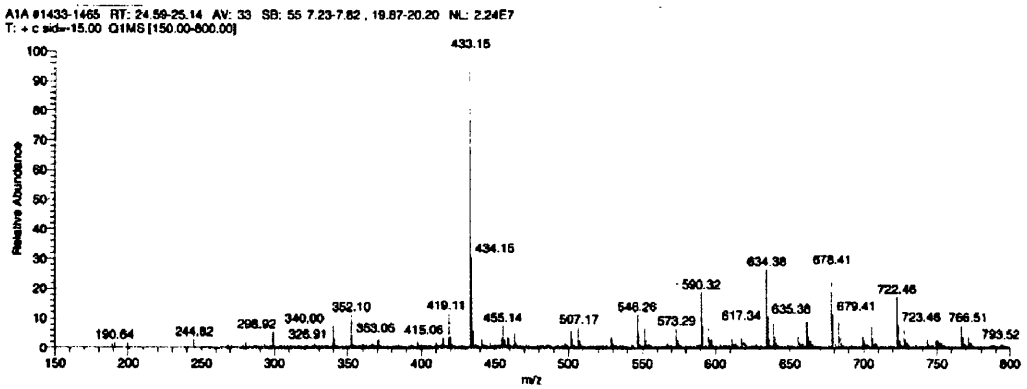


Figure 42.F: Mass spectrum of the compound eluting at 24.92 minutes.

ΔCYP158A2

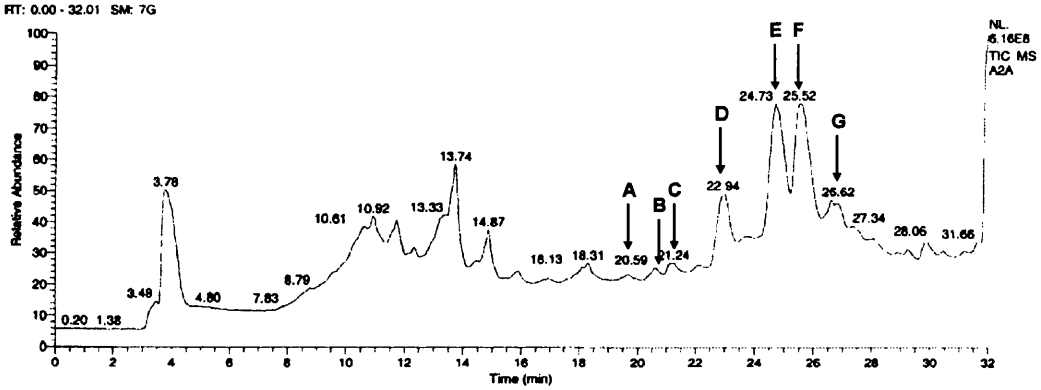


**Figure 43.** Total ion chromatogram (TIC) of the single mutant for CYP158A2 gene sample. The figures 43.A to 43.G show the mass spectra of the peaks labelled in the chromatogram.

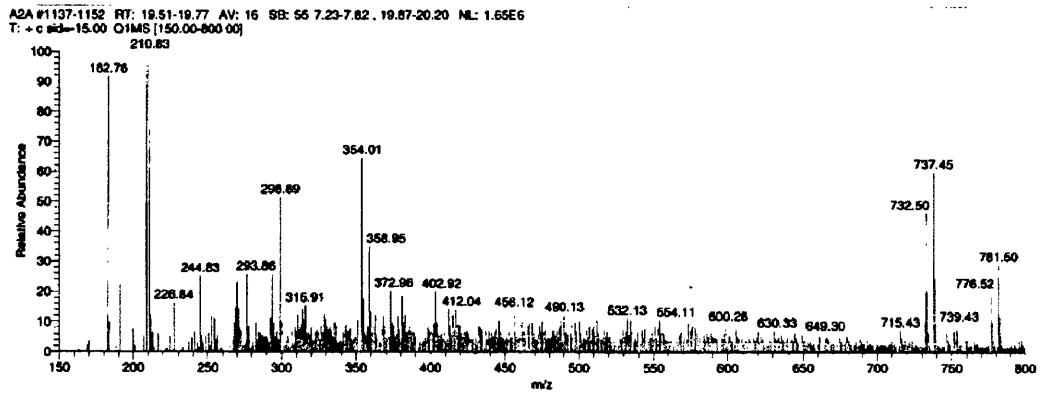


Figure 43.A: Mass spectrum of compound labelled as A.

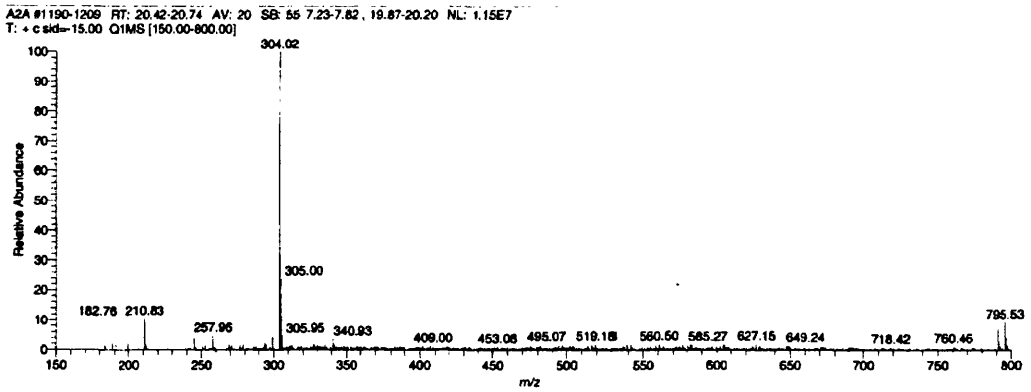


Figure 43.B: Mass spectrum of compound B.

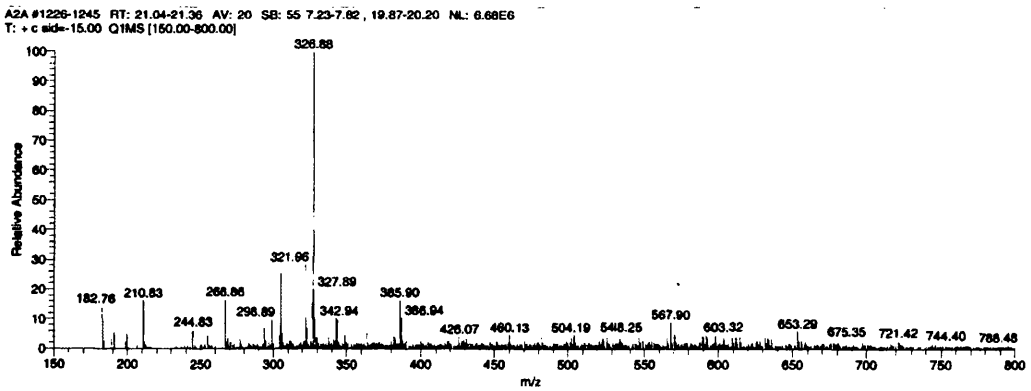


Figure 43.C: Mass spectrum of compound C.

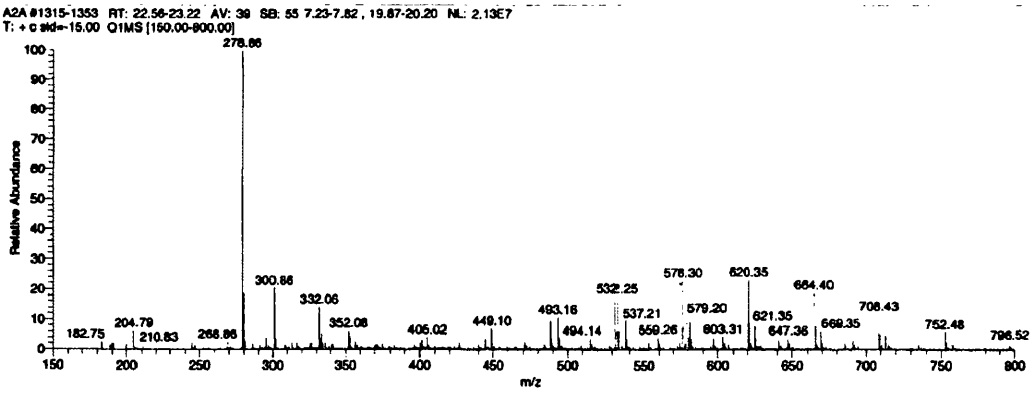


Figure 43.D: Mass spectrum of compound D.

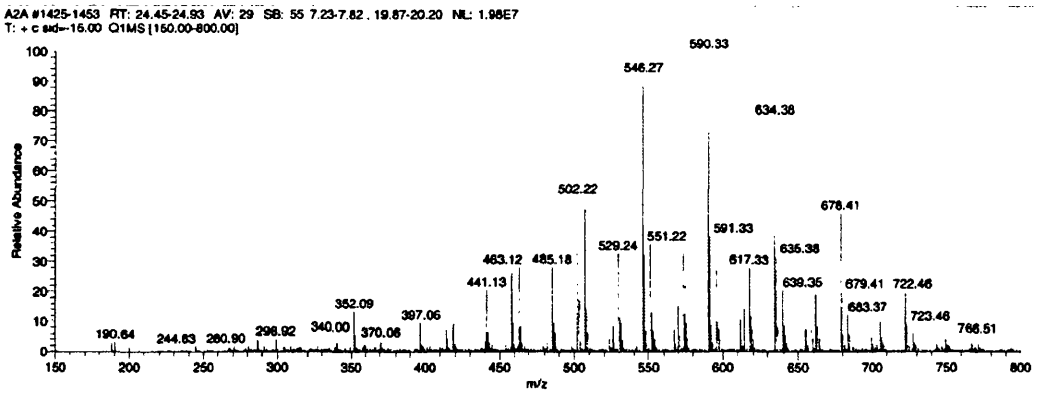


Figure 43.E: Mass spectrum of compound E.

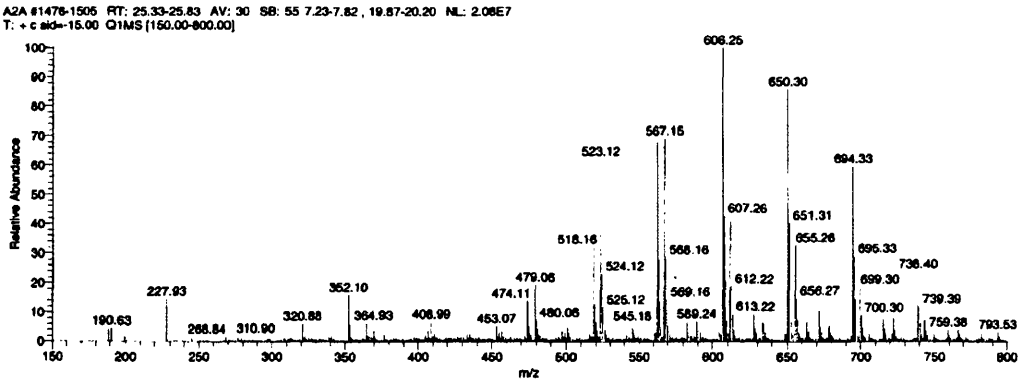


Figure 43.F: Mass spectrum of compound F.

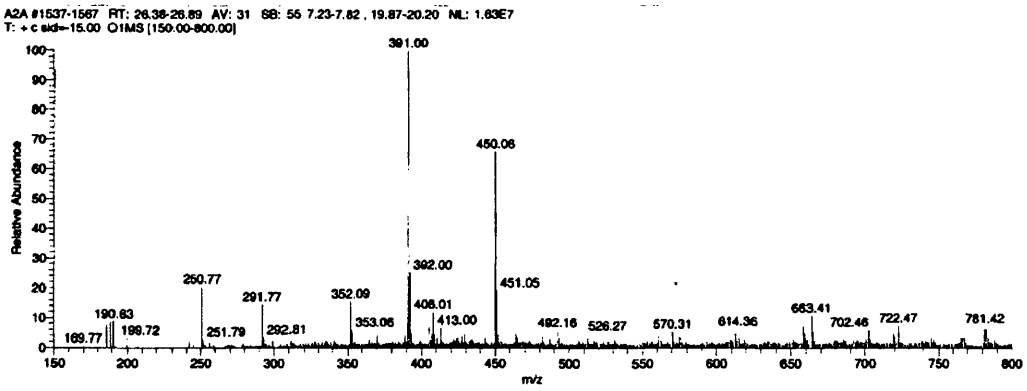


Figure 43.G: Mass spectrum of compound G.

ΔCYP158A1/A2

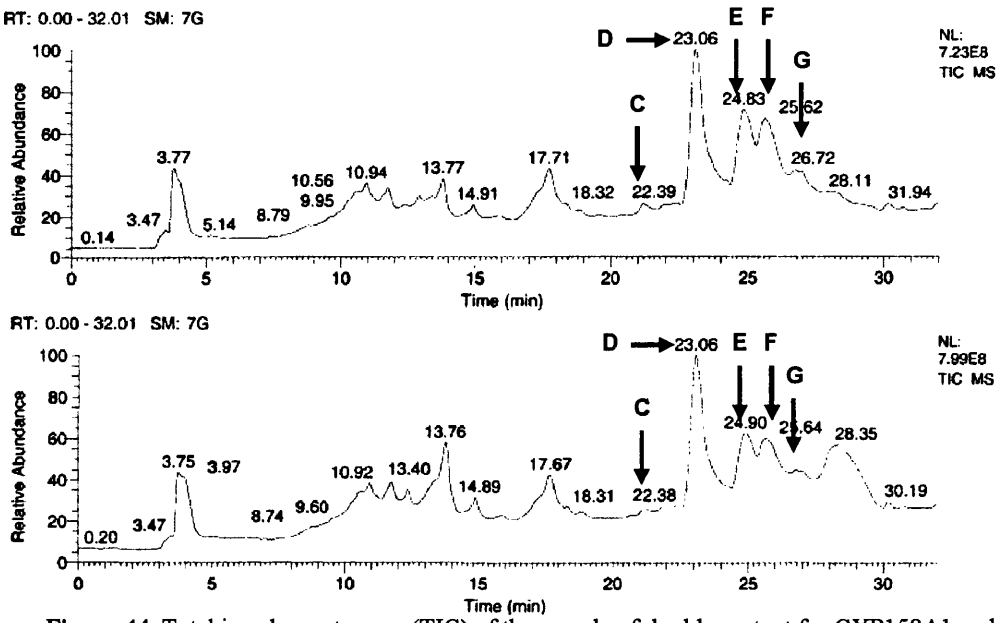


**Figure 44.** Total ion chromatogram (TIC) of the sample of double mutant for CYP158A1 and CYP158A2 genes. The peaks (compounds) obtained are the same than the ones obtained for the CYP158A2 single mutant. The mass spectra are shown in figures 43.A to 43.G.

None of the mass spectra obtained from the different compounds detected in each sample (parental strain, deletion mutant for CYP158A2 gene, deletion mutant for CYP158A1 gene, or deletion mutant for both genes) showed the typical flaviolin mass spectrum (figure 40).

Flaviolin is apparently not being accumulated in the cultures of the parental strain or any of the mutants.

**SUMMARY**

Deletion of the gene *sco6998* encoding CYP158A1 to create a single mutant and a double mutant for CYP158A1 and CYP158A2 genes was attempted following a PCR-based methodology. PCR screening and sequencing of the junctions between the inserted spectinomycin cassette and the adjacent regions of the locus *sco6998* were performed in order to investigate the gene replacement. The results suggest that the introduction of the spectinomycin cassette replacing the gene encoding for CYP158A1 were successful in three of the mutants screened, although this event was not confirmed by Southern Blot analysis so far.

No differences on phenotype were observed in the conditions tested between these mutants and the parental strain when culturing them in liquid and solid media. The mass spectra obtained from extracts of those cultures showed no resemblance in any case with the typical flaviolin spectra. However, it is noteworthy that the chromatographic profile of the single mutant for CYP158A1 was similar to that of the parental strain and the chromatographic profile of the single deletion mutant for CYP158A2 gene resembled that of the double mutant for both genes.

Deletion of the gene *sco6998* encoding for CYP158A1 in a single mutant decreased the viability of spores subjected to UV radiation treatment. Deletion of the gene encoding for CYP158A2 showed increased resistance of spores to the same UV treatment compared to the parental strain. The latter phenotype was also detected for the double mutant for both of these genes.

# 4 DISCUSSION

In actinomycetes PKS and their related cytochromes P450 are enzymes involved in synthesis and modification (respectively) of different polyketides. These modifications usually provide the special biological properties of these compounds (pigments, antibiotics, immunosuppressants).

CYP158A2 from *S. coelicolor* A3(2) is an enzyme with monooxygenase activity which is encoded within a three-gene operon together with a polyketide synthase (THN synthase) and ORF3. This THN synthase catalysis the formation of THN from five molecules of Malonyl-CoA, which is then oxidised to flaviolin. The role of the CYP is the biosynthesis of pigments for the protection against UV irradiation which achieves by oxidative coupling of two molecules of flaviolin.

CYP158A1 also uses flaviolin as substrate to yield the same products and it has been thought to be substituting CYP158A2 in function in a mutant with the gene for this enzyme deleted. The disruption of the gene *rppA*, an homologous of THN synthase of *S. coelicolor* in *Streptomyces griseus* and *Saccharopolyspora erythraea* led to the production of an albino phenotype. Given that, the same results were expected to be obtained when disrupting CYP158A2 gene (*sco1207*) or *rppA* (*sco1206*) in *S. coelicolor.* However, these gene knockouts resulted in no changes of phenotype (Izumikawa *et al.*, 2003;Funa *et al.*, 2005a;Zhao *et al.*, 2007). In the myxobacterium *Sorangium cellulosum,* a gene for a type III polyketide synthase was inactivated, and the mutant showed no physiological difference compared to the wild type strain (Gross *et al.*, 2006). This is why it has been suggested that these genes are silent, or that may not be expressed under normal growth conditions.

In *S. griseus*, RppA (THNS) is involved in production of HPQ-melanin, synthesized by the P450mel from THN. In other species THN is converted by MomA (quinone-forming monooxygenase) into its quinone, flaviolin, which is modificated by CYP158A2 in *S. coelicolor* to produce dimeric and trimeric red-brown pigments. In *S. erythraea* (red variant) flaviolin derivatives are synthesized to be incorporated to the red-brown pigment they typically produce.

The aim of this work was to try to corroborate the role of CYP158A1 as a substitute of CYP158A2 in the biosynthesis of those flaviolin-derived pigments. This hypothesis was tested by generation of two different mutants, a single mutant with the gene for the CYP158A1 disrupted and a double mutant for both CYP158A1 and A2. REDIRECT technology was used to this end as it is a well described and adapted method for *S. coelicolor*.

Typically, around 10% of the exconjugants obtained by the REDIRECT technology are double cross-over recombinants (Gust *et al.*, 2002). In fact, 12 out of 104 exconjugants obtained for the single mutation and 14 out of 116 for the double had the correct phenotype.

*Streptomyces* cosmids contain two different antibiotic resistance selective markers, an ampicillin resistance gene and a kanamycin resistance gene. Kanamycin resistance is usually used in these experiments as the selective marker. In this work, however, a high concentration of ampicillin, at which the parental strain does not grow, was used to screen exconjugants for the antibiotic resistance phenotype.

Southern hybridisation experiments were carried out with samples from four of those exconjugants in order to confirm the deletion of the gene *sco6998*. Conclusive results were not obtained in the time available by this method given that it was not possible to obtain enough signal for the control sample (parental strain DNA). The quantity of genomic DNA of the samples and the probe DNA was measured by spectrophotometry and proved to be sufficient. Its quality, measured as the ratio 260/280 was as well acceptable. Several variations were therefore done to the method used to try to optimise every condition to obtain a good signal in the membrane. However, none of these approaches was apparently enough to obtain a good resolution of bands.

*Bam*HI was the restriction enzyme used at the beginning of this work to digest the genomic DNA for the experiment of Southern hybridization. A *Bam*HI recognition site exists inside the gene *sco6998*. As this gene is cut during the digestion of the genomic DNA, the probability of hybridisation with the DNA probe

(which is the whole gene *sco6998*) is reduced. *Apa*I was therefore used as an alternative, more appropriate restriction enzyme to digest the genomic DNA. According to the restriction map obtained, this enzyme does not cut inside the gene nor the cassette producing a digestion fragment in the wild type of 1783 bp.

Depurination of the DNA in the agarose gel after electrophoresis and before washing it to transfer the DNA to the membrane through the Southern blot aids in the posterior denaturation step. However, this step should only be used when target DNA fragments exceed 15 kb (Sambrook and Russell, 2001). Given that our target DNA was 1783 bp long, that depurination step might be breaking the DNA molecules too much causing the fragments that go through to the membrane being too small to be retained (Sambrook and Russell, 2001). Therefore, after several vain attempts depurinating the DNA before the denaturation, this step was eliminated. This approach was nevertheless unsuccessful as detection of the bands was still not enough to obtain conclusive results.

Hybridisation temperature was as well varied to increase the probability of probe binding to the target DNA. Stringency washes were carried out at room temperature to avoid the loss of the DNA already hybridised. None of these procedures were sufficient to improve the signal in the membrane.

PCR screening was then performed to corroborate the mutation using different set of primers. The correct band pattern was detected for 2 of the mutants (10, single mutant for gene *CYP158A1*, and 13, double mutant for both genes *CYP158A1* and *CYP158A2*). Two bands of approximately 1600 and 1700 bp were observed in several lanes when using the flanking primers for the gene. The two bands correspond to both the gene (whose amplification product is theoretically 1650 bp long) and the spectinomycin cassette (whose amplification product should be 1851 bp long). These results suggest that both the gene and the disruption cassette are inside the chromosome and therefore a single cross-over occurred during recombination instead of the desired double cross-over in those mutants.

As a mean to verify if a single or double cross-over occurred during recombination, and therefore to confirm if the replacement of the gene took place,

PCR products from the putative mutants was sequenced. The sequence obtained for each of the samples was compared with both the chromosomal DNA sequence contained in the cosmid 8F11 and pIJ778 containing the spectinomycin cassette sequence.

Parental DNA was as well sequenced and used as a control sample. The DNA sequence at both sides of the *sco6998* locus indeed coincided with the chromosomal DNA sequence and not with the sequence contained in the plasmid pIJ778 as expected.

The DNA sequence obtained from the two fragments of DNA obtained when amplifying with the flanking primers for the gene *sco6998* of sample 3 (single mutant for gene *sco6998* encoding for CYP158A1) showed coincidence with both the chromosomal DNA contained in the cosmid 8F11 and the spectinomycin cassette sequence contained in the plasmid pIJ778. The largest band showed coincidence with the chromosomal DNA only in the region flanking the *sco6998* reading frame, and with the spectinomycin cassette sequence along the *sco6998* internal coding sequence. The smallest band showed no coincidence with the plasmid pIJ778 containing the spectinomycin cassette. These results suggest that this mutant possesses the spectinomycin cassette, as further confirmed by sequencing the band of DNA obtained with the internal primers for the cassette, but it also contains the gene *CYP158A1*. A single cross-over occurred during recombination of the disrupted cosmid with the chromosome of *S. coelicolor* instead of the desired double cross-over which would lead to replacement of the gene. Therefore the deletion in this mutant was not successful.

The same DNA sequence similarity analyses made for the rest of the mutants (samples 10, putative single mutant, 13 and 21, putative double mutants) at both sides of the PCR amplified fragments obtained for each sample, show how the insertion of the spectinomycin cassette was successful. When amplifying the DNA with a primer annealing inside the spectinomycin cassette, bands were obtained for the four of them, proving that the cassette was inserted in their chromosomes. Further sequencing of the DNA of those bands using this internal primer confirmed this fact, as the DNA sequence of the mutants coincides with the spectinomycin cassette

sequence in the region corresponding with the internal coding sequence of the gene *sco6998*. The sequence comparison of the DNA obtained by amplification with the flanking primers for the gene *sco6998* for all these samples reveals that the fragment amplified is not the gene *sco6998* encoding for CYP158A1 but the spectinomycin cassette. Moreover, in those mutants the flanking sequence adjacent to the inserted cassette matches the adjacent sequence to the gene. According to these results these three mutants seem to have the gene *sco6998* successfully deleted and replaced by the spectinomycin cassette.

Additional results from Southern Blotting experiments are necessary in order to confirm the double cross-over event which leads to the replacement of the gene. However, time limitations did not allow the completion of this procedure succesfully. The replacement of the gene *sco6998* by the spectinomycin cassette in these mutants is therefore still not completely proved.

In *S. griseus* P450mel is the protein responsible for the aryl coupling of THN to yield HPQ (1,4,6,7,9,12-hexahydroxyperylene-3,10-quinone) which polymerises to yield HPQ melanin (Funa *et al.*, 2005a). In *S. antibioticus* the protein encoded by the gene *momA* uses THN as substrate to yield flaviolin. In other *Streptomyces* species such as *Saccharopolyspora erythraea*, *S. avermitilis* or *S. coelicolor* the same gene organization has been found, including the gene *rppA* encoding for a type III PKS, a cytochrome P450 gene and a gene similar to *momA* all grouped in cluster. The aminoacid sequence of *momA* from *S. antibioticus* shares overall similarity to the ORF3 (*sco1208*) found in cluster with the THN synthase and CYP158A2 in *S. coelicolor* A3(2) (Funa *et al.*, 2005b).

A *P450mel* mutant of *S.griseus* showed the presence of a red pigment in the mycelium and spores of this organism, due to the accumulation of flaviolin produced from THN by autooxidation (Funa *et al.*, 2005a). The same phenotype was expected to be obtained in a CYP158A2 gene deletion mutant in *S. coelicolor* due to the accumulation of its substrate flaviolin. This deletion, however, produced no differences in the phenotype of this mutant compared to the wild type (Funa *et al.*, 2005b;Zhao *et al.*, 2007). CYP158A1 was thought to be substituting CYP158A2 in the formation of flaviolin derivatives given that it can catalyse oxidative coupling of

flaviolin to yield almost identical products. In this work the gene *sco6998* encoding for this CYP158A1 was investigated to prove this hypothesis in a single mutant and in a mutant for the CYP158A2 gene. These mutants were cultured in solid and liquid medium to screen any differences on the phenotype compared to the parental strain. However, the expected red pigment was not detected in the mycelium growing in liquid medium nor in the spores growing in MS plates, and any other significant change was detected.

The medium composition and culture conditions are critical factors in obtaining a desired phenotype. The lack of differences in phenotype of the mutants compared to the parental strain can be due to the fact that the conditions used in this experiment were not appropriate as reported previously for generating the phenotype for CYP158A2 mutant (Zhao *et al.*, 2007).

Apart from the genome and the proteome the study of the metabolome of an organism has become a useful tool to further characterise it. To this end, in this work Mass spectrometry experiments were carried out in order to characterise the mutants regarding the production or accumulation of flaviolin. The HPLC/MS experiments performed showed no detection of the flaviolin mass spectrum in the culture extracts of the parental strain or any of the mutants (single mutant for CYP158A1 gene, single mutant for CYP158A2 gene or double mutant for both genes). In the parental strain the flaviolin synthesised from THN is used by CYP158A2 and CYP158A1 to produce the flaviolin derived pigments. In the single mutants for the CYP158A1 or CYP158A2 genes flaviolin is also being used by one or the other CYP to yield those pigments. In the double mutant for both CYP158A1 and CYP158A2 genes, none of these CYPs are present to catalyse that flaviolin transformation. We assumed that the interruption of this pathway would lead to the accumulation of this metabolite. However, flaviolin was not detected in culture extracts of this mutant analysed by mass spectrometry. This suggests that flaviolin is not being accumulated or being converted into a different compound. It has been reported that flaviolin can be the precursor of other metabolites in other *Streptomyces* species. In *S. antibioticus* the product of the gene *momA* it has been seen to be involved in the modification of flaviolin to yield the pigment mompain (Funa *et al.*, 2005b). MomA shares overall similarity in amino acid sequence with the product of gene *sco1208*, also named

*ORF3*, which is located downstream adjacent to the gene encoding for CYP158A2 (*sco1207*) and included in a three-gene operon together with *sco1206* encoding for THN synthase. According to this, the gene product of *sco1208* might be involved in the utilisation of the flaviolin in the CYP158A1 and CYP158A2 double mutant.

Flaviolin derivatives are pigments for the protection against UV exposure in streptomycetes. Spore survival under UV radiation was measured to check the effect of the mutation on this feature. However the results of these experiments do not correspond to what was initially expected. Hypothetically, the double mutant with both genes for CYP158A1 and CYP158A2 deleted might not produce any of the flaviolin derivatives that protect this bacterium against ultraviolet radiation. Thus, this mutant would be expected to present less spore survival under the UV irradiation treatment than the wild type. However, the overall percentage of surviving spores after the treatment was higher than the percentage obtained for the wild type. The single mutant for the CYP158A1 gene was the only mutant which showed less spore resistance than the wild type, presenting approximately the same pattern along the time with dose response as the parental strain. This result coincides with the effect of the *P450mel* mutation in spore survival in *S. griseus*, where the deletion of this gene produced a reduction in the resistance to UV irradiation (Funa *et al.*, 2005a). The unexpected higher resistance observed in the spores of the CYP158A2 mutant and the double mutant for CYP158A1 and CYP158A2 genes, might suggest that CYP158A2 is involved in the production of a compound which potentiates UV effect on the spores of these bacteria or could be explained by the fact that in these mutants another compound is being produced which might enhance the resistance of these organisms against UV irradiation. This hypothesis agrees with the mass spectra of extracts of cultures of the single mutant for CYP158A2 and the double mutant. New compounds were detected for these samples that were not found in the extracts of the parental strain or the single mutant for CYP158A1.

Functionally related genes are often located in clusters in prokaryotes. The product of the gene *sco1208*, which is in cluster with the gene *sco1207* encoding for CYP158A2 and with the gene *sco1206* encoding for a THN synthase, shares overall amino acid sequence similarity with the product of the gene *momA* in *S. antibioticus*. MomA has been reported to be involved in the conversion of flaviolin to yield

mompain (Funa, 2005). Given that in a mutant for the gene *sco1207* there is no CYP158A2 to metabolise the flaviolin that is being produced from the THN synthesised by THN synthase, flaviolin might be being used by the *sco1208* gene product to yield a different compound, presumably providing higher resistance against UV irradiation to these bacteria. In a mutant for the CYP158A1 gene, flaviolin is being used by CYP158A2 to yield flaviolin derived pigments and therefore that compound is not being produced and is not providing that enhanced UV resistance.

## 4.1 FURTHER EXPERIMENTS

Confirmation of the desired mutation for the gene *sco6998* deletion by Southern blot is the first step that should be taken forward. Once the deletion is corroborated characterisation experiments on the phenotype of these mutants will shed light into the actual function of these proteins.

Optimization of Southern Blotting method is necessary to conclusively confirm the mutations. Several different approaches were done in this work to that end, none of them being successful. Other variations to the method that could be done are the transfer of the DNA to the membrane and the immunological detection. There are several immunological detection methods described, other than colorimetric detection by enzyme immunoassay using NBT/BCIP. One of them, which is probably more sensitive, is the use of chemiluminescent alkaline phosphatase substrates such as CDP or CSPD. When the alkaline phosphatase reacts with this compounds they produce light which is then detected exposing the membrane to a photographic film.

Once the replacement of the gene by the spectinomycin cassette has been confirmed, the REDIRECT technology culminates with the excision of the inserted disrupted cassette replacing the gene. This is achieved thanks to the FRT sites located at both ends of the cassette which will be recognised by the FLP-recombinase from *Saccharomyces cerevisiae*. The expression of this enzyme in *E. coli* removes the central part of the disruption cassette. However, not the whole cassette is

removed by this action and an 81 bp scar remains in the chromosome. This sequence must lack stop codons to maintain the reading frame.

The use of other methodologies to achieve the deletion of the gene should be considered. It has been previously reported that the REDIRECT technology was not successful on the deletion of several cytochromes P450 genes in their containing cosmids in *S. coelicolor* (Skaug, 2006). In this case, transposon mutagenesis was performed to create the disrupted cosmids.

Transposon mutagenesis consists of the replacement of a gene by a transposon. A *S. coelicolor* A3(2) ordered transposon library was constructed by Bishop, 2004 using transposon Tn5062, derived from Tn5, which carries an ampicilin resistance gene and a green florescent protein (GFP) as markers, and an *oriT* to allow the transfer into *S. coelicolor* by conjugation. A disrupted cosmid for the gene encoding the CYP158A1 from this library can be inserted in *S. coelicolor* and the positive clones selected in the same way as for the REDIRECT methodology. The success on this approach still depends on obtaining allelic exchange by double cross-over during recombination.

Characterisation of the mutants' phenotype will be essential to determine the effect of gene deletion. As well as optimising the culture conditions and media composition in the search of the expected phenotype of the mutants, mass spectrometry analyses will have to be performed again to determine their metabolic profile regarding flaviolin production, as well as ultraviolet irradiation assays which will show the effect of the lack of those flaviolin derived pigments on the survival of the spores.

Regardless of the method used to produce the gene disruption, when the mutants are found to have a different phenotype than the wild type strain, complementation of the mutation has to be performed in order to attribute that effect on the phenotype to that particular change on the phenotype. Complementation consists of the insertion of the gene back to the cell, generally by insertion of an expression plasmid containing the gene, and the restoring of the parental phenotype. Thus, if the parental phenotype is restored the change on phenotype can certainly be

attributed to that mutation, and the function of the corresponding protein is therefore elucidated.

Since the publication of *S. coelicolor* A3(2) CYPome in 2002, several of these proteins have been studied in order to know their function. Deletion of their genes has been achieved for some of them, and diverse studies to characterise these mutants and more gene disruptions for other CYPs will be done in the future. This work was an attempt to disrupt and characterise one of those 18 CYPs, in order to elucidate its function, and thus improve our knowledge about these diverse and valuable proteins.

Prenylation of small aromatic molecules is an important process leading to the biosynthesis of diverse secondary metabolites in bacteria. Flaviolin, THN, and other THN derivatives have been reported to be the substrate of several prenyltransferases catalysing the addition of isoprenoid groups to these molecules. Understanding the metabolic flux of these compounds in the pathway, as well as of pigments for the protection against UV radiation synthesised by CYP158A1 and CYP158A2 might mark the importance if this pathway in the identification of novel secondary metabolites.

**ANNEXE 1.** Primers used in this work

All the primers used in this work and their sequences are shown in the next table.

| **Primer Name** | **Sequence (5´to 3´)** | **Description** |
|---|---|---|
| 158A1conF1 | gtgcgctgatggtggagatccg | Amplification of the gene *sco6998* |
| 158A1conR1 | agggcctgatcggccagctgctg | |
| SpmCssF | gtataggaacttcgaagttcccgccagcctcgcagagcaggattc | Amplification of the spectinomycin cassette |
| SpmCssR | ctagagaataggaacttcggaataggaacttcatgagctc | |
| SpecIntF | ttatccagctaagcgcgaactgc | Internal primer for the spectinomycin cassette |

**ANNEXE 2.** Sequence of the plasmid pIJ778. (Obtained from (Gust *et al.*, 2002)

```
LOCUS pIJ778 4377 bp DNA CIRCULAR SYN 02-AUG-2002
DEFINITION Ligation of Spec,Strep-oriT disruption cassette into the EcoRV site of
pBluescript SK(+)
ACCESSION pIJ778
KEYWORDS .
SOURCE Unknown.
ORGANISM Unknown
Unclassified.
REFERENCE 1 (bases 1 to 4377)
AUTHORS Gust et al., 2003
JOURNAL Proc. Natl. Acad. Sci. USA 100(4), 1541-1546
FEATURES Location/Qualifiers
CDS 699..717
/region="priming site 19 bp"
/product="TGT AGG CTG GAG CTG CTT C"
CDS complement (718..751)
/region="FRT"
/product="natural FRT site"
CDS complement (798..1590)
/gene="aadA"
/product="spectinomycin,streptomycin adenyltransferase gene"
CDS 1931..2040
/region="oriT"
/product="origin of transfer (RK2)"
CDS 2049..2082
/region="FRT"
/product="natural FRT site"
CDS complement (2091..2110)
/region="priming site 20 bp"
/product="ATT CCG GGG ATC CGT CGA CC"
CDS complement (3386..4246)
/gene="amp"
/product="b-lactamase"
BASE COUNT 1065 a 1120 c 1108 g 1084 t
ORIGIN
1 GGAAATTGTA AACGTTAATA TTTTGTTAAA ATTCGCGTTA AATTTTTGTT AAATCAGCTC
61 ATTTTTTAAC CAATAGGCCG AAATCGGCAA AATCCCTTAT AAATCAAAAG AATAGACCGA
121 GATAGGGTTG AGTGTTGTTC CAGTTTGGAA CAAGAGTCCA CTATTAAAGA ACGTGGACTC
181 CAACGTCAAA GGGCGAAAAA CCGTCTATCA GGGCGATGGC CCACTACGTG AACCATCACC
241 CTAATCAAGT TTTTTGGGGT CGAGGTGCCG TAAAGCACTA ATCGGAACC CTAAAGGGAG
301 CCCCCGATTT AGAGCTTGAC GGGGAAAGCC GGCGAACGTG GCGAGAAAGG AAGGGAAGAA
361 AGCGAAAGGA GCGGGCGCTA GGGCGCTGGC AAGTGTAGCG GTCACGCTGC GCGTAACCAC
421 CACACCCGCC GCGCTTAATG CGCCGCTACA GGGCGCGTCG CGCCATTCGC CATTCAGGCT
481 GCGCAACTGT TGGGAAGGGC GATCGGTGCG GGCCTCTTCG CTATTACGCC AGCTGGCGAA
541 AGGGGGATGT GCTGCAAGGC GATTAAGTTG GGTAACGCCA GGGTTTTCCC AGTCACGACG
601 TTGTAAAACG ACGGCCAGTG AATTGTAATA CGACTCACTA TAGGGCGAAT TGGGTACCGG
661 GCCCCCCCTC GAGGTCGACG GTATCGATAA GCTTGATGTG TAGGCTGGAG CTGCTTCGAA
721 GTTCCTATAC TTTCTAGAGA ATAGGAACTT CGGAATAGGA ACTTCATGAG CTCAGCCAAT
781 CGACTGGCGA GCGGCATCTT ATTTGCCGAC TACCTTGGTG ATCTCGCCTT TCACGTAGTG
841 GACAAATTCT TCCAACTGAT CTGCGCGCGA GGCCAAGCGA TCTTCTTCTT GTCCAAGATA
901 AGCCTGTCTA GCTTCAAGTA TGACGGGCTG ATACTGGGCC GGCAGGCGCT CCATTGCCCA
961 GTCGGCAGCG ACATCCTTCG GCGCGATTTT GCCGGTTACT GCGCTGTACC AAATGCGGGA
1021 CAACGTAAGC ACTACATTTC GCTCATCGCC AGCCCAGTCG GGCGGCGAGT TCCATAGCGT
1081 TAAGGTTTCA TTTAGCGCCT CAAATAGATC CTGTTCAGGA ACCGGATCAA AGAGTTCCTC
1141 CGCCGCTGGA CCTACCAAGG CAACGCTATG TTCTCTTGCT TTTGTCAGCA AGATAGCCAG
1201 ATCAATGTCG ATCGTGGCTG GCTCGAAGAT ACCTGCAAGA ATGTCATTGC GCTGCCATTC
1261 TCCAAATTGC AGTTCGCGCT TAGCTGGATA ACGCCACGGA ATGATGTCGT CGTGCACAAC
1321 AATGGTGACT TCTACAGCGC GGAGAATCTC GCTCTCTCCA GGGGAAGCCG AAGTTTCCAA
1381 AAGGTCGTTG ATCAAAGCTC GCCGCGTTGT TTCATCAAGC CTTACGGTCA CCGTAACCAG
1441 CAAATCAATA TCACTGTGTG GCTTCAGGCC GCCATCCACT GCGGAGCCGT ACAAATGTAC
1501 GGCCAGCAAC GTCGGTTCGA GATGGCGCTC GATGACGCCA ACTACCTCTG ATAGTTGAGT
1561 CGATACTTCG GCGATCACCG CTTCCCTCAT GACATTGCAC TCCACCGCTG ATGACATCAG
1621 TCGATCATAG CACGATCAAC GGCACTGTTG CAAATAGTCG GTGGTGATAA ACTTATCATC
1681 CCCTTTTGCT GATGGAGCTG CACATGAACC CATTCAAAGG CCGGCATTTT CAGCGTGACA
1741 TCATTCTGTG GGCCGTACGC TGGTACTGCA AATACGGCAT CAGTTACCGT GAGCTGCATT
1801 TTCCGCTGCA TAACCCTGCT TCGGGGTCAT TATAGCGATT TTTTCGGTAT ATCCATCCTT
1861 TTTCGCACGA TATACAGGAT TTTGCCAAAG GGTTCGTGTA GACTTTCCTT GGTGTATCCA
1921 ACGGCGTCAG CCGGGCAGGA TAGGTGAAGT AGGCCCACCC GCGAGCGGGT GTTCCTTCTT
1981 CACTGTCCCT TATTCGCACC TGGCGGTGCT CAACGGGAAT CCTGCTCTGC GAGGCTGGCG
2041 GGAACTTCGA AGTTCCTATA CTTTCTAGAG AATAGGAACT TCGAACTGCA GGTCGACGGA
2101 TCCCCGGAAT ATCGAATTCC TGCAGCCCGG GGGATCCACT AGTTCTAGAG CGGCCGCCAC
2161 CGCGGTGGAG CTCCAGCTTT TGTTCCCTTT AGTGAGGGTT AATTCCGAGC TTGGCGTAAT
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2221 CATGGTCATA GCTGTTTCCT GTGTGAAATT GTTATCCGCT CACAATTCCA CACAACATAC
2281 GAGCCGGAAG CATAAAGTGT AAAGCCTGGG GTGCCTAATG AGTGAGCTAA CTCACATTAA
2341 TTGCGTTGCG CTCACTGCCC GCTTTCCAGT CGGGAAACCT GTCGTGCCAG CTGCATTAAT
2401 GAATCGGCCA ACGCGCGGGG AGAGGCGGTT TGCGTATTGG GCGCTCTTCC GCTTCCTCGC
2461 TCACTGACTC GCTGCGCTCG GTCGTTCGGC TGCGGCGAGC GGTATCAGCT CACTCAAAGG
2521 CGGTAATACG GTTATCCACA GAATCAGGGG ATAACGCAGG AAAGAACATG TGAGCAAAAG
2581 GCCAGCAAAA GGCCAGGAAC CGTAAAAAGG CCGCGTTGCT GGCGTTTTTC CATAGGCTCC
2641 GCCCCCCTGA CGAGCATCAC AAAAATCGAC GCTCAAGTCA GAGGTGGCGA AACCCGACAG
2701 GACTATAAAG ATACCAGGCG TTTCCCCCTG GAAGCTCCCT CGTGCGCTCT CCTGTTCCGA
2761 CCCTGCCGCT TACCGGATAC CTGTCCGCCT TTCTCCCTTC GGGAAGCGTG GCGCTTTCTC
2821 ATAGCTCACG CTGTAGGTAT CTCAGTTCGG TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG
2881 TGCACGAACC CCCCGTTCAG CCCGACCGCT GCGCCTTATC CGGTAACTAT CGTCTTGAGT
2941 CCAACCCGGT AAGACACGAC TTATCGCCAC TGGCAGCAGC CACTGGTAAC AGGATTAGCA
3001 GAGCGAGGTA TGTAGGCGGT GCTACAGAGT TCTTGAAGTG GTGGCCTAAC TACGGCTACA
3061 CTAGAAGGAC AGTATTTGGT ATCTGCGCTC TGCTGAAGCC AGTTACCTTC GGAAAAAGAG
3121 TTGGTAGCTC TTGATCCGGC AAACAAACCA CCGCTGGTAG CGGTGGTTTT TTTGTTTGCA
3181 AGCAGCAGAT TACGCGCAGA AAAAAAGGAT CTCAAGAAGA TCCTTTGATC TTTTCTACGG
3241 GGTCTGACGC TCAGTGGAAC GAAAACTCAC GTTAAGGGAT TTTGGTCATG AGATTATCAA
3301 AAAGGATCTT CACCTAGATC CTTTTAAATT AAAAATGAAG TTTTAAATCA ATCTAAAGTA
3361 TATATGAGTA AACTTGGTCT GACAGTTACC AATGCTTAAT CAGTGAGGCA CCTATCTCAG
3421 CGATCTGTCT ATTTCGTTCA TCCATAGTTG CCTGACTCCC CGTCGTGTAG ATAACTACGA
3481 TACGGGAGGG CTTACCATCT GGCCCCAGTG CTGCAATGAT ACCGCGAGAC CCACGCTCAC
3541 CGGCTCCAGA TTTATCAGCA ATAAACCAGC CAGCCGGAAG GGCCGAGCGC AGAAGTGGTC
3601 CTGCAACTTT ATCCGCCTCC ATCCAGTCTA TTAATTGTTG CCGGGAAGCT AGAGTAAGTA
3661 GTTCGCCAGT TAATAGTTTG CGCAACGTTG TTGCCATTGC TACAGGCATC GTGGTGTCAC
3721 GCTCGTCGTT TGGTATGGCT TCATTCAGCT CCGGTTCCCA ACGATCAAGG CGAGTTACAT
3781 GATCCCCCAT GTTGTGCAAA AAAGCGGTTA GCTCCTTCGG TCCTCCGATC GTTGTCAGAA
3841 GTAAGTTGGC CGCAGTGTTA TCACTCATGG TTATGGCAGC ACTGCATAAT TCTCTTACTG
3901 TCATGCCATC CGTAAGATGC TTTTCTGTGA CTGGTGAGTA CTCAACCAAG TCATTCTGAG
3961 AATAGTGTAT GCGGCGACCG AGTTGCTCTT GCCCGGCGTC AATACGGGAT AATACCGCGC
4021 CACATAGCAG AACTTTAAAA GTGCTCATCA TTGGAAAACG TTCTTCGGGG CGAAAACTCT
4081 CAAGGATCTT ACCGCTGTTG AGATCCAGTT CGATGTAACC CACTCGTGCA CCCAACTGAT
4141 CTTCAGCATC TTTTACTTTC ACCAGCGTTT CTGGGTGAGC AAAAACAGGA AGGCAAAATG
4201 CCGCAAAAAA GGGAATAAGG GCGACACGGA AATGTTGAAT ACTCATACTC TTCCTTTTTC
4261 AATATTATTG AAGCATTTAT CAGGGTTATT GTCTCATGAG CGGATACATA TTTGAATGTA
4321 TTTAGAAAAA TAAACAAATA GGGGTTCCGC GCACATTTCC CCGAAAAGTG CCACCTG
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# 5 BIBLIOGRAPHY

## 5.1 WEB SOURCES

Cytochrome P450 Homepage http://drnelson.utmem.edu/CytochromeP450.html
GenBank® http://www.ncbi.nlm.nih.gov/Genbank/index.html
NebCutter http://tools.neb.com/NEBcutter2/index.php
Oligo analyser http://www.basic.northwestern.edu/biotools/OligoCalc.html
Scripps Center for Mass Spectrometry http://masspec.scripps.edu/index.php
Streptomyces Annotation Server http://strepdb.streptomyces.org.uk
WatCut http://watcut.uwaterloo.ca/watcut/watcut/template.php

## 5.2 REFERENCE LIST


Alberts,B., Johnson,A., Lewis,J., Raff,M., Roberts,K., and Walter,P. (2008) Molecular Biology of the Cell Garland Science.

Barker,J. (1999) Mass Spectrometry John Wiley & Sons, LTD.

Bell,A.A., Stipanovic,R.D., and Puhalla,J.E. (1976) Pentaketide Metabolites of Verticillium-Dahliae - Identification of (+)-Scytalone as a Natural Precursor to Melanin. *Tetrahedron Tetrahedron* **32**: 1353-1356.

Bentley,S.D., Chater,K.F., Cerdeno-Tarraga,A.M., Challis,G.L., Thomson,N.R., James,K.D. *et al.* (2002) Complete genome sequence of the model actinomycete Streptomyces coelicolor A3(2). *Nature* **417**: 141-147.

Bruno,R.D., and Njar,V.C. (2007) Targeting cytochrome P450 enzymes: a new approach in anti-cancer drug development. *Bioorg Med Chem* **15**: 5047-5060.

Butz,D., Schmiederer,T., Hadatsch,B., Wohlleben,W., Weber,T., and Sussmuth,R.D. (2008) Module extension of a non-ribosomal peptide synthetase of the glycopeptide antibiotic balhimycin produced by Amycolatopsis balhimycina. *Chembiochem* **9**: 1195-1200.

Chakraburtty,R., and Bibb,M. (1997) The ppGpp synthetase gene (relA) of Streptomyces coelicolor A3(2) plays a conditional role in antibiotic production and morphological differentiation. *J Bacteriol* **179**: 5854-5861.

Chater,K.F. (2001) Regulation of sporulation in Streptomyces coelicolor A3(2): a checkpoint multiplex? *Curr Opin Microbiol* **4**: 667-673.

Chater,K.F. (2006) Streptomyces inside-out: a new perspective on the bacteria that provide us with antibiotics. *Philos Trans R Soc Lond B Biol Sci* **361**: 761-768.

Clarke,C.H., and Hopwood,D.A. (1976) Ultraviolet mutagenesis in Streptomyces coelicolor: induction of reversions in a polyauxotrophic strain. *Mutat Res* **41**: 201-208.

Cortes,J., Velasco,J., Foster,G., Blackaby,A.P., Rudd,B.A., and Wilkinson,B. (2002) Identification and cloning of a type III polyketide synthase required for diffusible pigment biosynthesis in Saccharopolyspora erythraea. *Mol Microbiol* **44**: 1213-1224.

Dahiya,J.S., and Rimmer,S.R. (1988) Accumulation of Flaviolin, 4-Hydroxyscytalone and 2-Hydroxyjuglone in Tricyclazole-Treated Cultures of Leptosphaeria-Maculans. *Phytochemistry* **27**: 3481-3482.

Datsenko,K.A., and Wanner,B.L. (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**: 6640-6645.

Deng,J., Carbone,I., and Dean,R.A. (2007) The evolutionary history of cytochrome P450 genes in four filamentous Ascomycetes. *BMC Evol Biol* **7**: 30.

Fiehn,O. (2002) Metabolomics--the link between genotypes and phenotypes. *Plant Mol Biol* **48**: 155-171.

Funa,N., Funabashi,M., Ohnishi,Y., and Horinouchi,S. (2005a) Biosynthesis of hexahydroxyperylenequinone melanin via oxidative aryl coupling by cytochrome P-450 in Streptomyces griseus. *J Bacteriol* **187**: 8149-8155.

Funa,N., Funabashi,M., Yoshimura,E., and Horinouchi,S. (2005b) A novel quinone-forming monooxygenase family involved in modification of aromatic polyketides. *J Biol Chem* **280**: 14514-14523.

Ghimire,G.P., Oh,T.J., Liou,K., and Sohng,J.K. (2008) Identification of a cryptic type III polyketide synthase (1,3,6,8-tetrahydroxynaphthalene synthase) from Streptomyces peucetius ATCC 27952. *Mol Cells* **26**: 362-367.

Girvan,H.M., Seward,H.E., Toogood,H.S., Cheesman,M.R., Leys,D., and Munro,A.W. (2007) Structural and spectroscopic characterization of P450 BM3 mutants with unprecedented P450 heme iron ligand sets. New heme ligation states influence conformational equilibria in P450 BM3. *J Biol Chem* **282**: 564-572.

Gross,F., Luniak,N., Perlova,O., Gaitatzis,N., Jenke-Kodama,H., Gerth,K. *et al.* (2006) Bacterial type III polyketide synthases: phylogenetic analysis and potential for the production of novel secondary metabolites by heterologous expression in pseudomonads. *Arch Microbiol* **185**: 28-38.

Gust,B., Chandra,G., Jakimowicz,D., Yuqing,T., Bruton,C.J., and Chater,K.F. (2004) Lambda red-mediated genetic manipulation of antibiotic-producing Streptomyces. *Adv Appl Microbiol* **54**: 107-128.

Gust,B., Kieser,T., and Chater,K.F. (2002) REDIRECT technology: PCR-targeting system in *Streptomyces coelicolor*. The John Innes Center, Norwich, United Kingdom.

Haagen,Y., Unsold,I., Westrich,L., Gust,B., Richard,S.B., Noel,J.P., and Heide,L. (2007) A soluble, magnesium-independent prenyltransferase catalyzes reverse and regular C-prenylations and O-prenylations of aromatic substrates. *FEBS Lett* **581**: 2889-2893.

Harold,R.J., and Hopwood,D.A. (1970) Ultraviolet-sensitive mutants of Streptomyces coelicolor. I. Phenotypic characterisation. *Mutat Res* **10**: 427-438.

Hesketh,A., Chen,W.J., Ryding,J., Chang,S., and Bibb,M. (2007) The global role of ppGpp synthesis in morphological differentiation and antibiotic production in Streptomyces coelicolor A3(2). *Genome Biol* **8**: R161.

Hoffmann,E.D., and Stroobant,V. (2001) Mass Spectrometry. Principles and applications John wiley & sons, LTD.

Holmes,D.S., and Quigley,M. (1981) A rapid boiling method for the preparation of bacterial plasmids. *Anal Biochem* **114**: 193-197.

Izumikawa,M., Shipley,P.R., Hopke,J.N., O'Hare,T., Xiang,L., Noel,J.P., and Moore,B.S. (2003) Expression and characterization of the type III polyketide synthase 1,3,6,8-tetrahydroxynaphthalene synthase from Streptomyces coelicolor A3(2). *J Ind Microbiol Biotechnol* **30**: 510-515.

Kelly,S.L., Lamb,D.C., Jackson,C.J., Warrilow,A.G., and Kelly,D.E. (2003) The biodiversity of microbial cytochromes P450. *Adv Microb Physiol* **47**: 131-186.

Kelly,S.L., Lamb,D.C., and Kelly,D.E. (2006) Cytochrome P450 biodiversity and biotechnology. *Biochem Soc Trans* **34**: 1159-1160.

Kiefer,P., Portais,J.C., and Vorholt,J.A. (2008) Quantitative metabolome analysis using liquid chromatography-high-resolution mass spectrometry. *Anal Biochem* **382**: 94-100.

Kieser,T., Bibb,M., Buttner,M.J., Chater,K.F., and Hopwood,D.A. (2000) Practical Streptomyces Genetics The John Innes Foundation, Norwich, United Kingdom.

Kuzuyama,T., Noel,J.P., and Richard,S.B. (2005) Structural basis for the promiscuous biosynthetic prenylation of aromatic natural products. *Nature* **435**: 983-987.

Lamb,D.C., Fowler,K., Kieser,T., Manning,N., Podust,L.M., Waterman,M.R. *et al.* (2002a) Sterol 14alpha-demethylase activity in Streptomyces coelicolor A3(2) is associated with an unusual member of the CYP51 gene family. *Biochem J* **364**: 555-562.

Lamb,D.C., Ikeda,H., Nelson,D.R., Ishikawa,J., Skaug,T., Jackson,C. *et al.* (2003) Cytochrome p450 complement (CYPome) of the avermectin-producer Streptomyces avermitilis and comparison to that of Streptomyces coelicolor A3(2). *Biochem Biophys Res Commun* **307**: 610-619.

Lamb,D.C., Skaug,T., Song,H.L., Jackson,C.J., Podust,L.M., Waterman,M.R. *et al.* (2002b) The cytochrome P450 complement (CYPome) of Streptomyces coelicolor A3(2). *J Biol Chem* **277**: 24000-24005.

Lamb,D.C., Waterman,M.R., Kelly,S.L., and Guengerich,F.P. (2007) Cytochromes P450 and drug discovery. *Curr Opin Biotechnol* **18**: 504-512.

Lewis,D.F., Watson,E., and Lake,B.G. (1998) Evolution of the cytochrome P450 superfamily: sequence alignments and pharmacogenetics. *Mutat Res* **410**: 245-270.

Mayerl,F., Piret,J., Kiener,A., Walsh,C.T., and Yasui,A. (1990) Functional expression of 8-hydroxy-5-deazaflavin-dependent DNA photolyase from Anacystis nidulans in Streptomyces coelicolor. *J Bacteriol* **172**: 6061-6065.

Nelson,D.R., and Strobel,H.W. (1987) Evolution of cytochrome P-450 proteins. *Mol Biol Evol* **4**: 572-593.

Oliver,S.G., Winson,M.K., Kell,D.B., and Baganz,F. (1998) Systematic functional analysis of the yeast genome. *Trends Biotechnol* **16**: 373-378.

Omura,T., and Sato,R. (1962) A new cytochrome in liver microsomes. *J Biol Chem* **237**: 1375-1376.

Ortiz de Montellano,P.R. (1995) Cytochrome P450 Plenum Publishing Corp., New Cork.

Plonka,P.M., and Grabacka,M. (2006) Melanin synthesis in microorganisms--biotechnological and medical aspects. *Acta Biochim Pol* **53**: 429-443.

Podust,L.M., Bach,H., Kim,Y., Lamb,D.C., Arase,M., Sherman,D.H. *et al.* (2004) Comparison of the 1.85 A structure of CYP154A1 from Streptomyces coelicolor A3(2) with the closely related CYP154C1 and CYPs from antibiotic biosynthetic pathways. *Protein Sci* **13**: 255-268.

Ringer,K.L., Davis,E.M., and Croteau,R. (2005) Monoterpene metabolism. Cloning, expression, and characterization of (-)-isopiperitenol/(-)-carveol dehydrogenase of peppermint and spearmint. *Plant Physiol* **137**: 863-872.

Ro,D.K., Paradise,E.M., Ouellet,M., Fisher,K.J., Newman,K.L., Ndungu,J.M. *et al.* (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* **440**: 940-943.

Romero-Martinez,R., Wheeler,M., Guerrero-Plata,A., Rico,G., and Torres-Guerrero,H. (2000) Biosynthesis and functions of melanin in Sporothrix schenckii. *Infect Immun* **68**: 3696-3703.

Rupasinghe,S., Schuler,M.A., Kagawa,N., Yuan,H., Lei,L., Zhao,B. *et al.* (2006) The cytochrome P450 gene family CYP157 does not contain EXXR in the K-helix reducing the absolute conserved P450 residues to a single cysteine. *FEBS Lett* **580**: 6338-6342.

Sambrook,J., and Russell,D.W. (2001) Molecular Cloning: A Laboratory Manual CSHL Press.

Schmid,E., Büchler,C., and Altenbuchner,J. (1999) AUD4, a new amplifiable element from Streptomyces lividans. *Microbiology* **145**: 3331-3341.

Skaug,T. (2006) Investigation of the cytochrome P450 complement in Streptomyces coelicolor A3(2) (PhD Thesis). University of Wales Aberystwyth.

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

Stumpe,M., and Feussner,I. (2006) Formation of oxylipins by CYP74 enzymes. *Phytochemistry Reviews* **5**: 347-357.

Takaya,N., Uchimura,H., Lai,Y., and Shoun,H. (2002) Transcriptional control of nitric oxide reductase gene (CYP55) in the fungal denitrifier Fusarium oxysporum. *Biosci Biotechnol Biochem* **66**: 1039-1045.

Taylor,M., Lamb,D.C., Cannell,R., Dawson,M., and Kelly,S.L. (1999) Cytochrome P450105D1 (CYP105D1) from Streptomyces griseus: heterologous expression, activity, and activation effects of multiple xenobiotics. *Biochem Biophys Res Commun* **263**: 838-842.

Tweeddale,H., Notley-McRobb,L., and Ferenci,T. (1998) Effect of slow growth on metabolism of Escherichia coli, as revealed by global metabolite pool ("metabolome") analysis. *J Bacteriol* **180**: 5109-5116.

Werck-Reichhart,D., and Feyereisen,R. (2000) Cytochromes P450: a success story. *Genome Biol* **1**.

Werck-Reichhart,D., Hehn,A., and Didierjean,L. (2000) Cytochromes P450 for engineering herbicide tolerance. *Trends Plant Sci* **5**: 116-123.

Werner,E., Heilier,J.F., Ducruix,C., Ezan,E., Junot,C., and Tabet,J.C. (2008) Mass spectrometry for the identification of the discriminating signals from metabolomics: current status and future trends. *J Chromatogr B Analyt Technol Biomed Life Sci* **871**: 143-163.

Wheeler,M.H., and Stipanovic,R.D. (1985) Melanin biosynthesis and the metabolism of flaviolin and 2-hydroxyjuglone in Wangiella dermatitidis. *Arch Microbiol* **142**: 234-241.

Wickramasinghe,R.H., and Ville,C.A. (1975) Early role during chemical evolution for cytochrome P450 in oxygen detoxification. *Nature* **256**: 509-511.

Williamson,N.R., Fineran,P.C., Leeper,F.J., and Salmond,G.P. (2006) The biosynthesis and regulation of bacterial prodiginines. *Nat Rev Microbiol* **4**: 887-899.

Zhao,B., Guengerich,F.P., Bellamine,A., Lamb,D.C., Izumikawa,M., Lei,L. *et al.* (2005) Binding of two flaviolin substrate molecules, oxidative coupling, and crystal

structure of Streptomyces coelicolor A3(2) cytochrome P450 158A2. *J Biol Chem* **280**: 11599-11607.

Zhao,B., Lamb,D.C., Lei,L., Kelly,S.L., Yuan,H., Hachey,D.L., and Waterman,M.R. (2007) Different binding modes of two flaviolin substrate molecules in cytochrome P450 158A1 (CYP158A1) compared to CYP158A2. *Biochemistry* **46**: 8725-8733.

Zhao,B., Lin,X., Lei,L., Lamb,D.C., Kelly,S.L., Waterman,M.R., and Cane,D.E. (2008) Biosynthesis of the sesquiterpene antibiotic albaflavenone in Streptomyces coelicolor A3(2). *J Biol Chem* **283**: 8183-8189.

Zhao,B., and Waterman,M.R. (2007) Novel properties of P450s in Streptomyces coelicolor. *Drug Metab Rev* **39**: 343-352.