Further Characterization of Dothistromin Genes in the Fungal Forest Pathogen *Dothistroma septosporum*

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ABSTRACT

Dothistroma septosporum is a forest pathogen that causes a disease called Dothistroma needle blight. The symptoms are thought to be due to the accumulation of dothistromin toxin produced by *D. septosporum*. Dothistromin is characterized as a difuranoanthraquinone and shows remarkable similarity to the aflatoxin (AF) and sterigmatocystin (ST) precursor versicolorin B. The similar structure to AF/ST suggests that dothistromin biosynthesis shares biosynthetic steps with the AF/ST pathway. The AF gene cluster in *Aspergillus parasiticus* and ST gene cluster in *A. nidulans* have been well characterized. Nine putative dothistromin biosynthetic genes have been identified. One of them, *dotA* was previously characterized by gene disruption and shown to have a similar function to homologous genes in AF/ST biosynthesis.

Two additional putative dothistromin biosynthetic genes, *pksA* and *epoA,* were characterized by gene disruption in this study. The inability of the *pksA* mutants to produce dothistromin indicated that the *pksA* is a key gene in dothistromin biosynthesis. The feeding of intermediates confirmed that *pksA* gene product is required for a very early step of dothistromin biosynthesis. The *pksA* mutants also showed reduced sporulation compared to wildtype, suggesting a relationship between dothistromin production and sporulation. The *epoA* gene replacements were also obtained successfully by homologous recombination. Both Southern blot and northern hybridization confirmed that the *epoA* gene was disrupted. However, the *epoA* mutants did not show any difference to the wild type in three analyses (growth rate, sporulation rate, dothistromin biosynthesis). However it was not possible to rule out a role for EpoA at a very late stage of dothistromin biosynthesis.

RACE analysis of the nine identified dothistromin genes characterized the transcription start and stop sites of the genes. Analyzing the putative regulatory protein binding motifs in the untranscribed region of the genes provided clues about the regulation of dothistromin biosynthesis and suggested there might be an *aflR*-like gene that governs dothistromin biosynthesis.

Both the *pksA* gene disruption and the RACE results suggested that the dothistromin biosynthetic pathway is homologous to that of AF/ST biosynthesis. Further work on the dothistromin gene cluster will help us to understand the evolution of fungal toxin gene clusters.

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Abbreviations

amp^r:	ampicillin resistance
bp:	base pair
cDNA:	complementary deoxyribonucleic acid
cm:	centimeter
°C:	degree celsius
CHEF:	contour-clamped homogeneous electric field
DNA:	deoxyribonucleic acid
dCTP:	deoxycytidine triphosphate
DEPC:	diethyl pyrocarbonate
DMSO:	dimethyl sulphoxide
DNase:	deoxyribonuclease
dNTP:	deoxynucleotide triphosphate
Fig:	figure
g :	gram
IPTG:	Isopropyl-β-d-thiogalactoside
kb:	kilobase pair
L:	litre
M:	mole per litre
ml:	milliliter
mM:	millimole per litre
OD_{600}	optical density at 600 nm
RNase:	ribonuclease
RNA:	ribonucleic acid
SDS:	sodium dodecyl sulfate
µl:	microlitre
µM:	micromole per litre
µg:	microgram
v/v:	volume per volume
w/v:	weight per volume
X-Gal:	5- bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER ONE: INTRODUCTION

1.1 *DOTHISTROMA* NEEDLE BLIGHT AND *DOTHISTROMA SEPTOSPORUM*

1.1.1 *Dothistroma* needle blight

Dothistroma septosporum is a forest pathogen, which causes a disease called *Dothistroma* needle blight. *Dothistroma* needle blight is a serious foliar disease in many species of *Pinus,* especially in *Pinus radiata* D. Don (Gilmour, 1967), which has played a major role in wood resources, particularly in New Zealand (Gibson, 1972). *P. radiata* covered up to 91% of the forest industry area and contributed 4% of the GDP for the country (based on 2002/2003, New Zealand statistics; http://www.nzforestry.co.nz/ nzf -content.asp?pageid=172).

The planting of single predominant forest species poses a high risk of serious outbreaks of pests and diseases. Unfortunately, *Dothistroma* needle blight has occurred on *P. radiata* in the countries of the southern hemisphere since the 1960s, such as in New Zealand, Australia, Chile and Kenya (Gibson, 1972; Ivory, 1967). Compared to the southern hemisphere, no large-scale outbreaks were recorded in the northern hemisphere, although there were some records of blight in *P. radiata* in Canada, California, Oregon and India (Gibson, 1972; Woods, 2003). Recently, however, a serious outbreak of *Dothistroma* needle blight has occurred in British Columbia, Canada. A local increase in summer precipitation appears to be responsible for the severe disease (Woods *et al.*, 2005).

1.1.2 *Dothistroma septosporum*

Identification of the pathogen of *Dothistroma* needle blight started in the 1900's. Until recently, both *Dothistroma pini* (Hulbary, 1941) and *D. septospora* (Morelet, 1968) were used as alternative names of the pathogenic fungus. Then, Barnes and co-workers analysed different isolates from 13 different countries by comparing their ITS, β-tublin and elongation factor 1-α gene sequence. They found that there were two species of *D. pini*. One is still called *D. pini*, which is restricted to the north-central U.S.A; and another is called *D. septosporum* (different from *D. septospora*) that has a

world distribution but is predominant in the southern hemisphere (Barnes *et al.*, 2004). The isolates from New Zealand belong to *D. septosporum*.

Only one strain of *D. septosporum* has been isolated in New Zealand so far. The isolates of the strain have been identified to have low diversity and asexual reproduction (Hirst *et al.*, 1999). Compared to the isolates from New Zealand, overseas isolates showed higher genetic diversity and some produce extremely high levels of dothistromin toxin (Bradshaw *et al.*, 2000).

1.1.3 The infection process

In order to find a way to control the disease, the infection conditions of *D. septosporum* have been investigated. The optimal infection conditions for the fungus were 20°C day/12°C night, continuous moisture, and high light intensity (Gadgil and Holden, 1976). The fungus usually enters the needles through stomata (Thyr and Shaw, 1964). The young *P. radiata* needles have larger stomatal pores than the needles of the mature pine and they are much more easily infected.

The *D. septosporum* infected needles contain necrotic lesions after 3.5 to 4 months, and brick-red bands appear (Gadgil, 1968). The red colour of the bands is caused by the accumulation of dothistromin toxin produced by *D. septosporum* (Shain and Franich, 1981). The survival period of *D. septosporum* in the infected needles is dependent on environmental conditions. Stromata of *D. septosporum* on suspended needles had a longer survival period than those in the litter; the longest period was 4 to 6 months in New Zealand. In east Africa, it was shown that, from 0°C to 37°C, the higher temperature and the drier condition, the shorter period the fungus could survive (Gibson, 1972).

1.1.4 Disease control

Currently, copper fungicides are still commonly used to control the disease, which is due to their ability to prevent germination of the fungal spores. Another way to control the disease is by breeding *Dothistroma* resistant cultivars of *P. radiata*. For

septosporum. Five genes were identified in the λ KSA clone: *pks A* (Jin, 2005; Seconi, 2001), *cypA, avfA, epoA* (Teddy, 2004) and *moxA* gene (Jin, 2005; Teddy, 2004).

BLASTX analysis of the dothistromin gene sequences showed that in the identified dothistromin cluster genes, except the *epoA* gene, all others have a putative homolog in the AF/ST cluster, as shown in Fig 1.2a. The putative dothistromin genes and their homologs in *A. parasiticus* and *A. nidulans* are shown in Table 1.1. The function of the AF/ST homologs is also shown.

In the identified dothistromin genes, *dotA* was the only gene that had been characterized by gene disruption prior to this project (Bradshaw *et al.*, 2002). The *dotA* mutant produced undetectable levels of dothistromin (tested by ELISA) and accumulated versicolorin A, which suggested that *dotA* is involved in dothistromin biosynthesis and also has the same function as the *aflM* gene in *A. parasiticus* (Bradshaw *et al.*, 2002). This result provided experimental evidence that the dothistromin biosynthetic pathway is similar to that of AF/ST and also confirmed that the AF/ST gene cluster is very helpful for identifying dothistromin genes.

Intriguingly, the binding site for an important regulatory protein, AflR, in AF biosynthesis (Chang *et al.*, 1995), 5'-TCGN$_5$CGR-3', is found upstream of six of nine identified dothistromin genes. Furthermore the sequence 5'-TCGN$_{11}$CGR-3, an expanded AflR-like binding site, is found in five of the nine putative dothistromin genes. Binding sites for another transcription factor, AbaA (that regulates expression of structural genes involved in conidiation and other developmental processes) (Ehrlich *et al.*, 2002) are also seen in seven dothistromin genes. Furthermore, two global regulator sites, PacC (the pH regulatory protein) and AreA (a regulator in nitrogen metabolism) (Calvo *et al.*, 2002), are also identified in these genes. PacC binding sites (5'-GCCARG-3') occur upstream of all the identified dothistromin genes and there are putative AreA binding sites present in seven of them.

The transcriptional start and stop sites for the dothistromin genes have not been defined. Some of the putative regulatory protein binding sites may be located in the transcribed but untranslated regions (UTR) of these genes. The RACE (Rapid Amplification cDNA Ends) method provides a means to identify both ends of the

gene (5'and 3' untranslated region). The results will help to determine which the putative regulatory motifs are. RACE analysis will also shed a light on the organization of genes in the cluster. For example the putative translation stop sites of *epoA* and *avfA* are only 250 bp apart and there is a possibility of overlapping transcripts.

Fig 1.2 Comparison between dothistromin, aflatoxin and streigmatocystin gene clusters

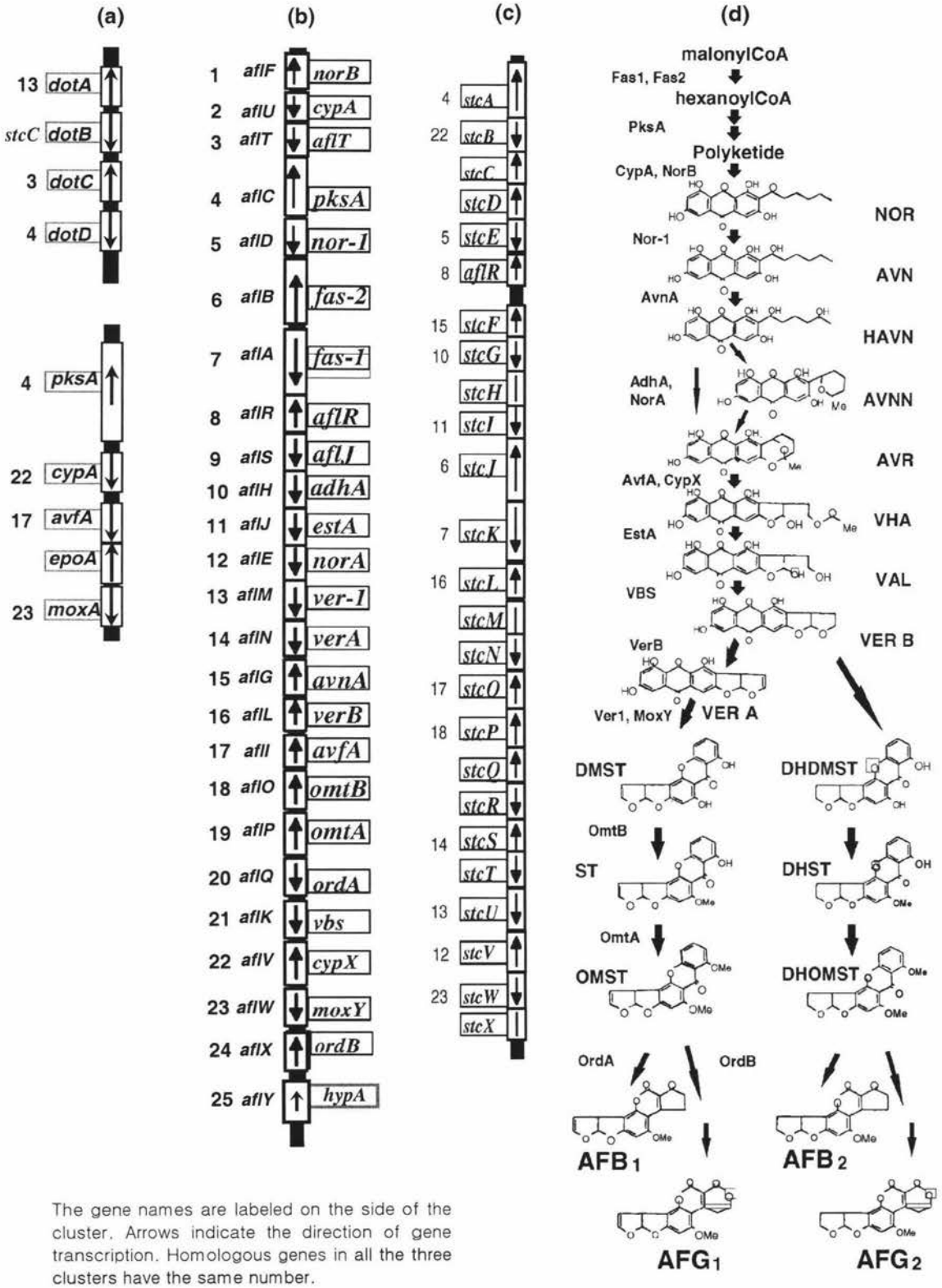
a. Fragments of the dothistromin gene cluster identified so far.

b. AF biosynthetic pathway gene cluster in *A. parasiticus* and *A. flavus*. The recently reclassified names (*afl*) are shown alongside the original names in the AF pathway gene cluster. (Bhatnagar *et al.*, 2003; Yu *et al.*, 2004a)

c. ST biosynthetic pathway gene cluster in *A. nidulans*. The gene names are labeled on the side of the cluster. Homologous genes in both clusters have the same number (Bhatnagar *et al.*, 2003).

d. Biosynthetic steps of aflatoxin. *AFB*$_1$ Aflatoxin B_1, *AFB*$_2$ aflatoxin B_2, *AFG*$_1$ aflatoxin G_1, *AFG*$_2$ aflatoxin G_2, *AVN* averantin, *AVNN* averufanin, *AVR* averufin, *DHDMST* dihydrodemethylstengmatocystin, *DHOMST* dihydro-*O*-methylsterigmatocystin, *DHST* dihydrosterigmatocystin, *DMST* demethylsterigmatocystin, *HAVN* 5'-hydroxyaverantin, *NOR* norsolorinic acid, *OMST O*-methylsterigmatocystin, *VAL* versiconal, *VERA* versicolorin A, *VERB* versicolorin B, *VHA* versiconal hemiacetal acetate (Bhatnagar *et al.*, 2003)

Clustered genes for dothistromin (a), AF (b), ST (c) and the AF biosynthetic pathway (d)



The gene names are labeled on the side of the cluster. Arrows indicate the direction of gene transcription. Homologous genes in all the three clusters have the same number.

dotB gene is homologous to *stcC* but has no homolog in the AF cluster.

b, c, d are modified from (Bhatnagar *et al.*, 2003; Yu *et al.*, 2004b)

Table 1.1 Putative dothistromin genes and their homologs in the AF/ST gene cluster

Dothistromin gene name (current)	Homolog		AF/ST Protein homology	#The step of AF biosynthesis	Reference(s)
	A. parasiticus (AF)	*A. nidulans* (ST)			
***dotA**	***aflM**	***stcU**	Dehydrogenase	Convert VERA to DMST	Bradshaw *et al.*, 2002
***dotB**	_	***stcC**			Bradshaw *et al.*, 2002
***dotC**	***aflT**	_	MFS transporter		Bradshaw *et al.*, 2002
***dotD**	***aflC (TE)**	***stcA(TE)**	TE domain of Polyketide synthase	Convert hexanoylCoA to dodecaketide	Bradshaw *et al.*, 2002
***pksA**	***aflC**	***stcA**	Polyketide synthase		(Morgan, 1997) Seconi, 2001 Jin, 2005
***cypA**	***aflV**	***stcB**	P450mono oxygenase	Convert AVR to HVN	Seconi, 2001
***avfA**	***aflI**	***stcO**	Oxidoreductase	Convert AVR to VHA	Teddy, 2004
***moxA**	***aflW**	***stcW**	Monooxygenase	Convert AVR to HVN	Teddy, 2004 Jin, 2005
***epoA**	_	_			Teddy, 2004

HVN: 1-hydroxyversicolorone; AVR, DMST, VERA and VHA are the same abbreviations as in Fig 1.2d.

Shows the step that the dothistromin gene homolog in *A. parasiticus* and /or *A. nidulans* is involved in.

* Genes whose function was characterized by gene disruption.

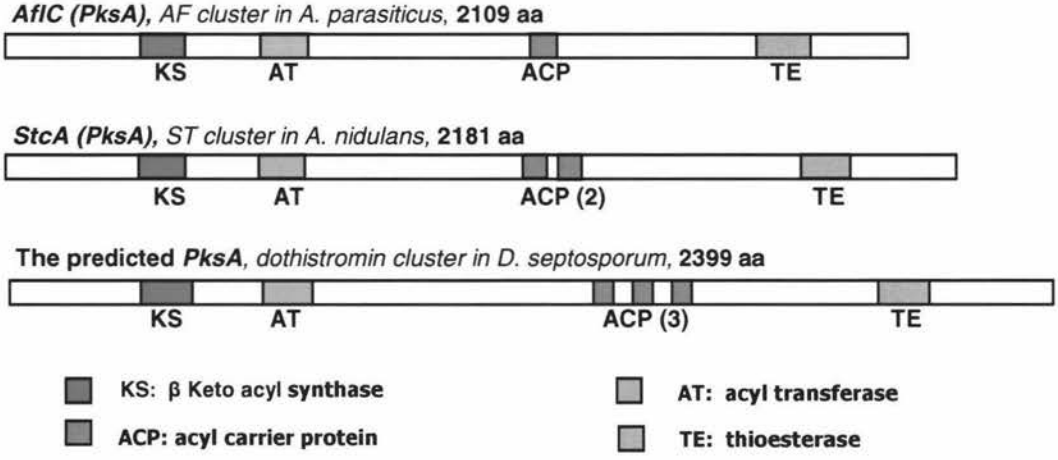
1.2.5 The putative *pksA* gene

As shown in Fig 1.2d, the *pksA* gene is a key gene in the early steps of AF/ST biosynthesis. It encodes an enzyme called polyketide synthase (PKS), which can use short-chain fatty acid hexanoylCoA as a starter unit to assemble the carbon chain for AF/ST.

The PKS enzymes are classified into two categories based on how close they are to the architecture of type I (vertebrate) or type II (bacterial and plant) fatty acid synthases (Hopwood and Sherman, 1990). Fungal PKSs belong to type I. All fungal PKSs contain β-ketoacyl synthase (KS), acyl transferase (AT) and acyl carrier protein (ACP) domains. Some of them also contain dehydratase (DH), enoyl reductase (ER), keto reductase (KR), thioesterase (TE) and another ACP domain. The AF *aflC (pksA)* gene product contains KS, AT, ACP and TE domains (Feng and Leonard, 1995) . The ST *stcA* gene (Yu and Leonard, 1995) product is similar but contains two ACP domains instead of one in AF *aflC* gene.

The putative *pksA* gene in *D. septosporum* was predicted to encode a polypeptide of 2399 amino acids (aa) and contains six conserved domains: KS, AT, three ACP and one TE. Three ACP domains are unusual in fungal PKSs. The *pksA* gene is the largest compared to its AF/ST homologs. A comparison of PKS proteins from *A. parasiticus* and *A. nidulans* with the predicted PksA from *D. septosporum* is shown in Fig 1.3.

Fig 1.3 The comparison of PKS proteins from *A. parasiticus, A. nidulans* to the predicted PksA from *D. septosporum*



The function of the AF and ST *pks* genes, *aflC and stcA*, were characterized by gene disruption. There was no detectable AFB1 expressed in an *aflC* mutant (Feng and Leonard, 1995) or ST in a *stcA* mutant (Yu and Leonard, 1995). So the function of the *pksA* gene is very interesting. Whether it is really involved in the initial step of dothistromin biosynthesis and has the same function as *aflC* and *stcA* in the AF/ST cluster still needs to be characterized by gene disruption. Furthermore, a mutant of *D. septosporum* that is blocked at a very early stage of dothistromin biosynthesis will be useful in further studies to determine whether dothistromin is a pathogenicity determinant.

1.2.6 The putative *epoA* gene

The putative *epoA* gene is located between two putative dothistromin genes, *avfA* and *moxA*. The *avfA* homolog, *aflI*, is involved in converting AVR to VHA (Table 1.1) in AF biosynthesis. The *moxA* homolog, *stcW* is required to convert AVR to HVN and the *cypA* homolog, *stcB,* functions at the same step as *stcW* in ST biosynthesis. Thus the *epoA* gene is surrounded by AF/ST-like genes even though it does not itself have a homolog in the AF/ST clusters. Whether it is a dothistromin biosynthetic gene and how it functions is very interesting.

The *epoA* gene open reading frame (ORF) is 1314 bp in length, contains one intron and encodes a 438 aa protein. The deduced amino acid sequence was analysed by BLASTP. It showed 45% identity to epoxide hydrolase (EH) in *A. niger* and 44% identity to a hypothetical protein AN3107.2 in *A. nidulans* FGSC A4 (this hypothetical protein gene is not located on the same chromosome as the ST cluster genes). The EH gene in *A. niger* was cloned and well characterized. There were 9 exons and 8 introns in the *A. niger* EH gene sequence. The three-domain structure of EH in *A. niger* has been resolved by X-ray crystallography. The result showed that the EH structure in *A. niger* has a strong similarity to that of human microsomal EH (Zou *et al.*, 2000).

EH enzymes are unique enzymes that catalyze the hydrolysis of epoxides to their vicinal diols without a cofactor (Smit, 2004). EHs belong to a large super family of

enzymes, the α/β-hydrolase fold enzymes, and contain a core domain, a β-sheet packed between two layers of 7α-helices and a cap domain of 5 α-helices. EHs have been widely found in organisms, such as mammals, invertebrate, plants, fungi and bacteria. Some of them are involved in the synthesis of secondary metabolites whilst others are involved in detoxification (Barth *et al.*, 2004).

The deduced amino acid sequence of EpoA has a conserved EHN (epoxide hydrolase N terminus) domain at the N-terminus. Whether it also has the other common EH structural components require further analysis. There is no EH gene involved in AF/ST biosynthesis. Whether it is involved in dothistromin biosynthesis and what the function of *epoA* is in *D. septosporum* will be characterized by disruption of the *epoA* gene.

1.3 GENE DISRUPTION

Gene disruption is an efficient way to study gene function. It has been employed to characterize gene function in the AF/ST cluster. For example, for *aflC* (Feng & Leonard 1995), *aflV* (*cypX*) and *aflW* (*moxY*) (Wen *et al.*, 2005) in *A. parasiticus*; and *stcE* in *A. nidulans* (Butchko *et al.*, 1999). It also has been widely used in plant pathogens, such as characterization of a trypsin protease gene function in a tomato pathogen *Verticillium dahliae* (Dobinson *et al.*, 2004); *CPS1* gene (encoding a protein with two AMP-binding domains) function in *Cochliobolus heterostrophus* (maize pathogen), *C. victoriae* (oats pathogen) and *Gibberella zeae* (wheat pathogen) (Lu *et al.*, 2003) and malate synthase gene (*mls1*) in wheat pathogen *Stagonospora nodorum* (Solomon *et al.*, 2004). In the dothistromin cluster, the *dotA* gene function was also determined by gene disruption (Bradshaw *et al.*, 2002).

Generally, there are three steps involved in gene disruption. Firstly, a construct of a gene disruption vector containing a selectable marker gene needs to be prepared. The dominant selectable marker, *E. coli* hygromycin B phosphotransferase gene (*hph*), conferring resistance to the eukaryotic antibiotic hygromycin B, is commonly used. Secondly, the construct needs to be transformed into the fungus. Thirdly, after transformation, the transformants need to be screened and characterized. In the following sections these steps will be considered separately.

1.3.1 Gene disruption construct

A popular method for gene replacement involves using a selectable marker gene to replace part of the target gene region. This can be achieved by a general method with PCR, restriction enzyme digestion and ligation, or with a relatively new technique called Gateway cloning system (Sasaki et al., 2004). For the first method, restriction enzyme cutting sites are very important for vector construction. Often, it is not very easy to find a suitable enzyme. The Gateway system employs special signal sequences (*att* sites) in the vector, along with enzymatic machinery to construct vectors through homologous recombination at the *att* sites, without using restriction endonucleases and ligases (Sasaki *et al.*, 2004).

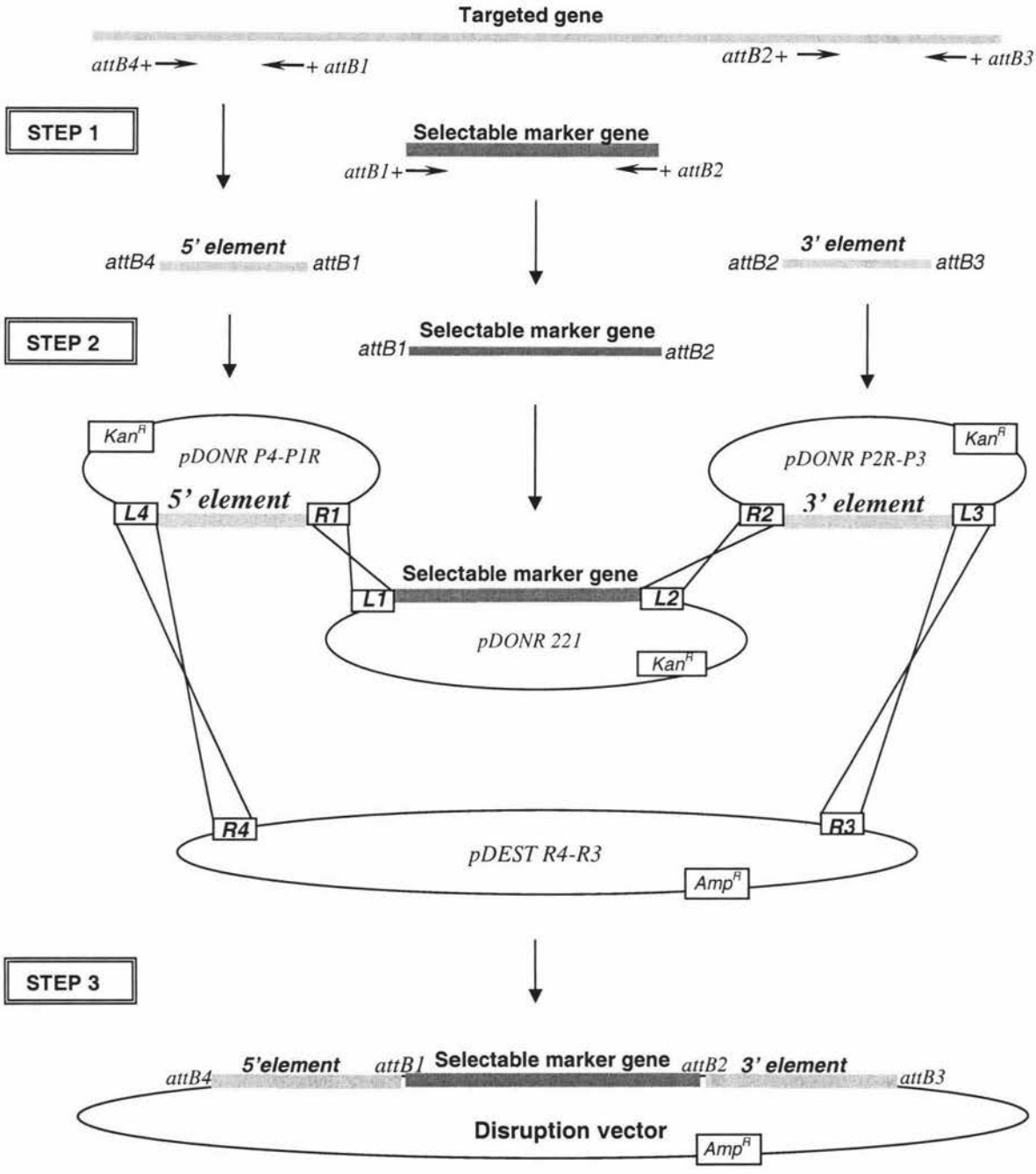
A general 3-step scheme to prepare a gene disruption construct using the Gateway system is shown in Fig 1.4. Firstly, the proper *att* sites are added to the cloned gene regions by PCR; then the PCR products are used to construct three entry clones with a special enzyme by recombination. Finally, these entry clones are constructed into a destination vector by recombination with another recombinase enzyme to obtain the disruption vector.

The advantage of the Gateway system is that it makes cloning more flexible because there is no need to consider restriction endonuclease cutting sites for the ligation reaction. The enzymes used in steps 2 and 3 recognize the specific *att* sequences and then join them together. Therefore, this method was employed in this project.

1.3.2 Fungal transformation methods

Once the construct is ready, it needs to be transformed into the host cells and a suitable transformation system needs to be chosen. Currently, there are several strategies employed in the transformation of filamentous fungi. Traditionally, PEG induced fusion of protoplasts has been widely used in the presence of transforming DNA. Alternatively, electroporation can be used to transform filamentous fungi (Chakraborty and Kapoor, 1990). More recently, *Agrobacterium tumefaciens* mediated transformation, widely used in plant transformation, was also extended to

Fig 1.4 Schematic diagram for Gateway system



→ Primer and primer direction, the primer arrows indicate the approximate location of the primers used for amplifying the 5'and 3'element.

filamentous fungi (de Groot *et al.*, 1998). These three methods are usually all protoplast-mediated procedures, but the latter two methods have greater transformation efficiency than the traditional one (Chakraborty and Kapoor, 1990; de Groot *et al.*, 1998).

There is an additional method called Biolistic (biological ballistic) transformation (Lorito *et al.*, 1993), which can directly transfer foreign DNA into host conidia. The advantages of this method are that protoplast preparation is not required and stable transformants can be obtained directly from the transformation plate, particularly if uninucleate spores are used that do not require subsequent purification (Te'o *et al.*, 2002).

However, all of these transformation strategies have to be optimized when employed with a new species for the first time. The protoplast/PEG method has been optimized (Bradshaw *et al.*, 1997) and used for characterization of the *dotA* gene function in *D. septosporum* (Bradshaw *et al.*, 2002). So for the *pksA* and *epoA* gene disruption, this method will still be used for transformation.

1.3.3 Identification and purification of mutants

The vector DNA used for transformation can be circular or linear. Once the DNA has been transferred into the host cells, it will be integrated in the host genome resulting in stable transformants. These tranformants can be identified, in this case, resistance to hygromycin, that is maintained even after growth on non-selective media. The gene disruption is achieved by homologous recombination. However, most of the integrations are ectopic; usually only a small percentage occurs at the homologous locus. In the case of the gene disruption construct where there are two homologous regions flanking a selectable marker gene, homologous recombination may occur at both homologous regions. In this case, part of the targeted gene (between the two homologous regions) will be replaced by the *hph* selectable marker gene. Hence, the transformants need to be screened to determine which kind of integration event has taken place.

Protoplasts made from mycelia are multinucleate; therefore the initial transformants are likely be heterokaryons (Fincham, 1989). This problem can be easily solved by plating conidia and isolating single colonies. After three rounds of single spore purification, the probability of loss of one or other nuclear component is high (Fincham, 1989). The *dotA* gene mutants were purified in this way (Bradshaw *et al.*, 2002).

The purified mutants will be screened by PCR and confirmed by Southern blotting. PCR can distinguish gene replacements and ectopic transformants. Southern blotting can be used to confirm the PCR results, identify the mutants (loss of wild type band) and provide information about the vector integration copy numbers.

1.3.4 Characterization of the mutants

Once a mutant is identified, its function needs to be characterized. As mentioned above, *pksA* is predicted to encode a multifunctional PKS enzyme and *epoA* is predicted to encode an EH enzyme that can be involved both in detoxification and/or secondary metabolism. Whether mutants of these two genes have any effects on dothistromin production and whether they accumulate any particular intermediates need to be tested. Dothistromin production will be quantified by competitive enzyme-linked immuno-adsorbent assay (ELISA) and the intermediates accumulation will be checked by thin-layer chromatography (TLC).

Secondary metabolism is commonly associated with sporulation in fungi (Calvo *et al.*, 2002). The relationship between asexual sporulation (conidiation) and the production of the secondary metabolite ST was tested for four different ST cluster gene mutants, Δ*aflR* (no ST intermediates), Δ*stcE* (no ST intermediates), Δ*stcJ* (accumulated NOR) and Δ*stcU* (accumulated versicolorin A). The results showed that the conidia (asexual spore) production was correlated with each progressive step in ST biosynthesis. The further along the ST pathway, the greater the number of conidia produced; such as Δ*stcJ* strains that showed greater conidiation than Δ*stcE* strains (Wilkinson *et al.*, 2004). In view of this work it is intended to assess sporulation of *D. septosporum* *pksA* and *epoA* mutants.

1.4 AIMS AND OBJECTIVES

The main aim of this research was to further characterize genes in the dothistromin gene cluster; in particular the *pksA* and *epoA* genes, and to carry out RACE analysis of all identified putative dothistromin biosynthetic genes. There were three objectives within the aim.

The first objective was to characterize the *pksA* gene function in dothistromin biosynthesis. The *pksA* mutants were obtained by transforming a disruption vector into *D. septosporum*, and confirmed by Southern hybridization. A series of comparisons between the mutants and the WT were carried out to check the effect of the *pksA* gene function on dothistromin biosynthesis, growth and sporulation. Finally, the *pksA* gene function was confirmed by intermediate feeding.

The second objective was to characterize the *epoA* gene function in *D. septosporum* and determine if *epoA* is involved in dothistromin biosynthesis. This was achieved by constructing a disruption vector and subsequent transformation into *D. septosporum*. Mutants were identified, confirmed and characterized. Finally, *epoA* gene expression in the mutants was checked by northern hybridization.

The third objective was to identify the 5'and 3'end UTRs of all nine dothistromin genes using RACE assays. The start and stop sites could be clarified and the gene regulatory motifs and organization of genes within the cluster could be analyzed.

CHAPTER TWO: MATERIALS AND METHODS

2.1 FUNGAL AND BACTERIAL STRAINS, LAMBDA CLONES AND PLASMIDS

Fungal and bacterial strains and plasmids used in this study are listed in Table 2.1. All the media, buffers and solutions are listed in Appendices I and II.

2.2 GROWTH AND MAINTENANCE OF CULTURES

2.2.1 Growth and Maintenance of *E. coli* Cultures

E. coli cultures were grown at 37°C overnight on LB agar plates, or in LB broth, with the appropriate selection supplements. Plates were stored at 4°C. The selected transformed *E. coli* (overnight inoculated LB broth) were also mixed with sterile glycerol at an *E. coli* to glycerol ratio of 17:3 for storage at –80°C.

2.2.2 Growth and Maintenance of *D. septosporum* Cultures

2.2.2.1 Single Spore Purification

In order to obtain a genetically pure colony of *D. septosporum*, single spores were isolated and grown as follows. A sterile loop of MilliQ water was wiped over a sporulating colony (growing on DSM) to collect spores. This loop was then streaked across a new DSM plate (selective in the case of transformants), the plate was turned and streaked again from the previous streak 3 times, flaming the loop between streaks. This plate was grown up until a single colony was a sufficient size to repeat the streaking process. After two streaked plates a single colony was isolated and said to be pure.

2.2.2.2 Growth of *D. septosporum* from transformed protoplasts

D. septosporum protoplasts were embedded in 5 ml molten DM top agar and poured onto plates containing osmotically stabilised 10 ml DM Suc. Selective plates had 70 µg/ml hygromycin (final concentration) in 5 ml DM top agar added 24 hours after embedding of protoplasts. Protoplasts were grown for approximate 7-14 days at 22°C. The putative transformants were subcultured and grown on selective DM plates for 2 weeks before subculturing onto selective DSM plates to induce sporulation.

Table 2.1 Plasmids, fungal and bacterial Strains.

Strains /plasmid	Relevant Characteristics	Source or Reference
Fungal Strains		
Dothistroma septosorum		
NZE 7(wild type)	Single spore isolate, laboratory strain.	(Barron, N. personal communication)
NZE 8(wild type)	Single spore isolate, laboratory strain.	
***EpoA* transfromants**		
E4 (*epoA* mutant)	*epoA*::*hph*, derivative of NZE7	This study
E6 (*epoA* mutant)	*epoA*::*hph*, derivative of NZE7	This study
E18(*epoA* mutant)	*epoA*::*hph*, derivative of NZE7	This study
E19(*epoA* mutant)	*epoA*::*hph*, derivative of NZE7	This study
E21(*epoA* mutant)	*epoA*::*hph*, derivative of NZE7	This study
E1 (ectopic)	*epoA*::*hph*, derivative of NZE7	This study
E10 (ectopic)	*epoA*::*hph*, derivative of NZE7	This study
E11 (ectopic)	*epoA*::*hph*, derivative of NZE7	This study
***pksA* transformants**		
P1-7(*pksA* mutant)	*pksA*::*hph*, derivative of NZE7	This study
P4 (*pksA* mutant)	*pksA*::*hph*, derivative of NZE7	This study
P6 (*pksA* mutant)	*pksA*::*hph*, derivative of NZE7	This study
P8 (*pksA* mutant)	*pksA*::*hph*, derivative of NZE7	This study
P12 (*pksA* mutant)	*pksA*::*hph*, derivative of NZE7	This study
P16 (*pksA* mutant)	*pksA*::*hph*, derivative of NZE7	This study
P42 (*pksA* mutant)	*pksA*::*hph*, derivative of NZE7	This study
P44 (*pksA* mutant)	*pksA*::*hph*, derivative of NZE7	This study
P1-8 (*pksA* mutant)	*pksA*::*hph*, derivative of NZE8	This study
P53 (*pksA* mutant)	*pksA*::*hph*, derivative of NZE8	This study
P58 (*pksA* mutant)	*pksA*::*hph*, derivative of NZE8	This study
P9 (ectopic)	*pksA*::*hph*, derivative of NZE7	This study
P10 (ectopic)	*pksA*::*hph*, derivative of NZE7	This study
P19 (ectopic)	*pksA*::*hph*, derivative of NZE8	This study
P83 (ectopic)	*pksA*::*hph*, derivative of NZE8	This study
P85 (ectopic)	*pksA*::*hph*, derivative of NZE8	This study
***Escherichia coli* strains**		
XL1-Blue	SupE44 hsdR17 recA1 endA1 gyrA46 *thi rel* A1 lac$^-$ F' [proAB$^+$ lacI^q Δ (lacZ) M15 Tn10 (tet^r)]	(Bullock *et al.*, 1987)
Top10	F$^-$ *mcr* A Δ(*mrr-hsd* RMS-*mcr* BC) Φ80 (*lacZ*)ΔM15 Δ *lac* X74 *rec* A1 *deo*R *ara*D139 Δ(*ara-leu*)7697	Invitrogen, Carlsbad, CA

Strains /plasmid	Relevant Characteristics	Source or Reference
Plasmids		
pGEM-T Easy	Amp^r, lacZ' (3.0 kb)	Promega, Madison, WI, USA
pGEM-T	Amp^r, lacZ' (3.0 kb)	Promega
pDONR P4-P1R	Cm^R, $Kanamycin^R$, *ccdB* gene (4.8kb)	Invitrogen
pDONR P2R-P3	Cm^R, $Kanamycin^R$, *ccdB* gene (4.8kb)	Invitrogen
pDEST R4-R3	Cm^R, $Ampicillin^R$, *ccdB* gene (4.4kb)	Invitrogen
pAN7-1	Hyg^R Amp^R (6.8kb)	(Punt and van den Hondel, 1992)
pR225	*hph* gene in pDNOR 221	(Teddy, 2004)
pR226	*pksA* disruption plasmid	(Teddy, 2004)
pR246	*epoA* disruption plasmid	This study

2.2.2.3 General growth and maintenance of *D. septosporum* cultures

Generally, *D. septosporum* was grown on DM or DSM plates at 22°C in the dark for 14-21days, with (mutants) or without (WT) the appropriate selection supplements. Plates were stored at 4°C. The selected *D. septosporum* mutants' mycelia were stored in 17% sterile glycerol in H_2O at -80°C.

2.3 DNA ISOLATION, PURIFICATION AND QUANTIFICATION

2.3.1 Small scale genomic DNA isolation from *D. septosporum* cultures

This method provides a simple and efficient way to prepare genomic DNA from filamentous fungi. In the lysis buffer DNA is separated from polysaccharides by mild shearing and NaCl used to precipitate polysaccharides and protein. The modification of the method is that fresh mycelia are directly used instead of dry mycelia (Al-Samarrai and Schmid, 2000).

Approximately 0.5 cm × 0.5 cm area of fresh mycelia from a plate culture was ground with a sterile plastic pestle in a microcentrofuge tube. Freshly prepared lysis buffer (500 µl) was added and mixed with vigorous pipetting. Two µl of 10 mg/ml RNAseA was added and the mixture was incubated for 5 minutes at 37°C. Cellular debris was precipitated by adding 165 µl of 5 M NaCl and mixed by inversion. The mixture was then centrifuged at 4°C (15,700 × g, in a microcentrifuge) for 10 minutes. The supernatant was transferred immediately into a fresh microcentrifuge tube. One volume of chloroform was added and mixed by inverting the tubes until the solution became milky. The mixture was centrifuged as before for 10 minutes. The aqueous (top) phase was removed to a fresh tube. One volume of isopropanol was added and centrifuged at 15,700 × g for 5 minutes. The pellet was washed three times with 500 µl of 70% ethanol, dried and resuspended in 100 µl of sterile MilliQ H_2O.

2.3.2 Large scale DNA isolation from *D. septosporum* cultures

CTAB (hexadecyltrimethylammonium bromide) DNA extraction method (Doyle and Doyle, 1987) was developed for isolating plant DNA. It has been successfully used for preparation of *D. septosporum* genomic DNA for Southern hybridization (Jin, 2005). So,

this method was used to prepare genomic DNA for Southern hybridization in this project.

Approximately 0.3-0.5 g freeze-dried mycelia were ground to fine powder with liquid nitrogen in a sterile mortar and pestle. This was distributed to microcentrifuge tubes with ~100 mg per tube. CTAB buffer (600 µl) and 2 µl of RNAseA (10 mg/ml) were added to the tubes and mixed thoroughly, then incubated at 37°C for 10 minutes; followed by incubation at 65°C for 1 hour. After incubation, 600 µl chloroform was added to the tube and mixed thoroughly, then centrifuged at 15,700 × g for 5 minutes. After centrifugation, the upper phase was transferred to a new microcentrifuge tube, and a further 600 µl chloroform again, and mixed thoroughly, then centrifuged at 15,700 × g for 5 minutes. The upper phase was transferred to another new microcentrifuge tube; one volume isopropanol was added to the tube and the tube inverted several times to mix. The mixture was incubated at room temperature for 2 minutes, centrifuged at full speed for 2 minutes. The pellet was washed three times in 1 ml 70% ethanol. After the last wash; ethanol was removed then the microcentrifuge tube left to air-dry, then the DNA was resuspended in sterile MilliQ H_2O.

2.3.3 Isolation of Plasmid DNA from *E. coli*

Plasmid DNA was isolated from *E. coli* using a QIAGEN plasmid minikit (≤ 20 µg plasmid DNA) or QIAGEN plasmid midikit (≤ 100 µg plasmid DNA) according to the manufacturers' instructions.

2.3.4 Agarose Gel Purification of DNA fragments

DNA containing the fragment to be purified was run on a 1-1.5% agarose gel in 1x TBE buffer. After staining in ethidium bromide, the DNA was visualised under long wave UV light (366nm), and the appropriate band excised using a clean scalpel blade. The DNA was extracted from the agarose using the QIAquick Gel extraction kit (QIAGEN) according to the manufacturers' instructions.

2.3.5 Purification of PCR Products from a PCR Reaction

DNA was purified from a PCR reaction either by gel purification (Section 2.3.4), or the QIAquick PCR purification Kit (QIAGEN) according to the manufacturers' instructions.

2.3.6 Purification of DNA by phenol/chloroform Extraction

TE buffer was added to DNA solution to make a volume of 500 µl, and 500 µl of phenol/chloroform/isoamyl alcohol (25:24:1) was added and mixed by inversion, then centrifuged for 10 minutes at 15,700 × g. The aqueous phase was transferred to a new microcentrifuge tube and 500 µl of chloroform was added to the solution, mixed and centrifuged at 15,700 × g for 10 minutes. The upper phase was transfer to another new microcentrifuge tube. DNA was precipitated by adding 1/10 volume of 3 M sodium acetate, and 1 volume of isopropanol. The mixture was incubated at room temperature for 2 minutes, centrifuged at full speed for 2 minutes. The pellet was washed once in 1 ml 70% ethanol. The ethanol was removed and the microcentrifuge tube was left to air-dry, then the DNA was resuspended in sterile MilliQ H_2O.

2.3.7 Agarose Gel electrophoresis of DNA

DNA fragments were size fractionated by electrophoresis through 0.7%-2.0% agarose dissolved in 1 × TBE buffer at 5-8 volts / cm (1.5-2 volts/cm for Southern blot). Dyes (xylenecyanoFF and bromophenol) present in the gel loading buffer, which was mixed with DNA samples before loading, allowed estimation of DNA migration. When the purple dye (xylenecyanoFF) shifted to ~1.5 cm to the edge of the gel, then the gel was ready. After electrophoresis, agarose gels were stained in ethidium bromide for 10-20 minutes, washed in water, observed under short wave UV (254 nm) and photographed.

2.3.8 Determination of DNA concentration by fluorometric assay

Fluorometric quantification was used for pure DNA samples. DNA was quantified on a Hoefer DyNA Quant 200 Flurometer (Amersham Bioscience) according to the manufacturer's protocol. The scale of the Fluorometer was set to 100 using 2 µl of 100 ng /µl calf thymus DNA added to 2 ml of a dye solution containing 1 × TNE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA and 100 mM NaCl, pH 7.4) and 0.1 µg/ml Hoechst 33258 dye. Once the scale was reliably set, 2 µl of sample DNA was added to 2 ml of the dye solution, and the resulting value was recorded as the concentration of DNA in ng/µl.

2.3.9 Determination of DNA concentration by Gel electrophoresis

Determination of DNA concentration by gel electrophoresis was performed by running a series of Lambda DNA or pUC118 DNA concentration standards on an agarose gel

alongside the DNA sample. The DNA sample concentration was estimated by comparing the intensity of ethidium bromide fluorescence to that of the DNA concentration standards.

2.3.10 Determination of DNA molecular Weights

DNA fragments were sized by running the DNA sample on an agarose gel alongside known size 1 kb^+ ladder (Invitrogen). The mobility of the standard markers from the wells were measured and used to determine the molecular weight of the unknown fragments by comparing the relative mobility between the lanes.

2.4 DNA DIGESTION, LIGATION AND CLONING

2.4.1 Restriction endonuclease Digestion of DNA

Restriction digests were performed at 37°C, in the buffer specified by the manufacturer, normally for 1 hour, but overnight for Southern blots. Digestion was performed with 1-10 units of restriction enzyme per μg DNA. RNA was removed by the addition of RNAse A to a final concentration of 1.0 μg/ml prior to incubation. Digestions were checked by running an aliquot on an agarose gel (Section 2.3.7).

2.4.2 Standard ligation Reactions

(pGEM-T and pGEM-T Easy vectors)

PCR products to be cloned were purified (Section 2.3.5) usually by PCR column or gel purification, then ligated into pGEM-T and pGEM-T Easy vector and transformed into a suitable *E. coli* host strain (Section 2.5.1). Blue/white colour selection was used for initial screening of transformants. When numbers of suspected recombinant transformants were large, PCR (Section 2.6.2) with appropriate primers was performed to screen transformants. The presence of insert was always confirmed by restriction enzyme digestion (Section 2.4.1) or PCR (Section 2.6.2) with isolated plasmid DNA (Section 2.3.3).

Ligation reactions were set up in 10 μl with the required vector: insert ratio and final concentration of 1 × ligation buffer and 0.5 to 1 μl of 3 u/μl T4 ligase. Ligation

reactions were mixed well and incubated at 4°C overnight. Ligation mixtures and controls were used to transform *E. coli* (Section 2.5.1).

2.4.3 Gateway™ Recombination Reactions

(Using the MultiSite Gateway™ Three-Fragment Vector Construction Kit, Invitrogen)

2.4.3.1 BP recombination

In previous research (Teddy, 2004), it was found that recombination between circular DNA (containing *attB* sites) and the donor vector is more efficient than between linear DNA (PCR products with *attB* sites) and the donor vector. So, PCR products containing the 3' or 5'element (homologous region) of the gene to be replaced and *attB* sites were cloned into pGEM-T Easy vector prior to the BP recombination reaction. The BP reaction was set up according to the MultiSite Gateway™ Three-Fragment Vector Construction Kit manual instruction (Invitrogen) using 100 fmol (40 ng) *attB* PCR product of 5' or 3'element added to 1µl (60 ng) appropriate pDONR vector along with 2 µl 5 × BP clonase reaction buffer and 2 µl BP clonase enzyme mix with TE buffer in a reaction volume of 10 µl. The mixture was briefly vortexed and centrifuged then incubated at 25°C overnight. The recombination reactions were cleaned using a PCR column, and then transformed into Top 10 competent cells by electroporation (Section 2.5.1.1) or chemical transformation (Section 2.5.1.2).

2.4.3.2 LR recombination

After BP recombination, the three entry clones contained LR sites, which are used for LR recombination with a DESTINATION vector. The LR recombination was used to create a final disruption vector. The reaction set up was also according to the manufacturer's instructions. Approximate 60 ng of each entry clone was mixed with 60 ng of DESTINATION vector along with 4 µl 5 × LR clonase reaction buffer and 4 µl LR clonase enzyme mix and added TE buffer to a final volume 20 µl. The mixture was briefly vortexed and centrifuged then incubated at 25°C overnight. The recombination reactions were cleaned using a PCR column and then transformed into Top 10 competent cells by electroporation (Section 2.5.1.1) or chemical transformation (Section 2.5.1.2).

The use of proteinase K was suggested by the manufacturer to inactivate the BP (or LR) clonase enzyme, which can affect the transformation. However, a PCR column was expected to have an even better function than proteinase K, as well as inactivating the clonase, it can also clean up the protein from the reaction system. This hypothesis needs to be proved. So, a comparison was made of transformation frequency (both with chemical and electroporation transformation) between proteinase K and PCR column clean up methods.

For checking the transformation efficiency, 1 µl of the reaction mixture was directly transformed into Top10 *E. coli* competent cells by electroporation (Section 2.5.1.1) or chemical transformation (Section 2.5.1.2). When appropriate 0.5 µl proteinase K was added to 5 µl of the reaction mixture, incubated at 37 °C for 1 hour, and then 1 µl of the proteinase K treated reaction mixture was used for electroporation (Section 2.5.1.1) or for chemical transformation (Section 2.5.1.2) into Top10 *E. coli* competent cells. Ten µl of the reaction mixture was cleaned up using a PCR column, and 2 µl of the cleaned reaction mixture was transformed into Top10 *E. coli* competent cells by electroporation (Section 2.5.1.1) or chemical transformation (Section 2.5.1.2).

2.5 TRANSFORMATION PROTOCOL

2.5.1 Transformation *of E. coli* competent Cells

2.5.1.1 Preparation of competent cells and transformation by electroporation

One litre of XL-1 blue (or Top10) *E. coli* cell culture (OD_{600} = 0.5-1.0) was chilled on ice for 20 minutes and the cells harvested by centrifugation at 4,080 × g (5000 rpm, GSA) for 10 minutes at 4°C. The cells were washed twice with 1 L then 0.5 L of ice-cold water by centrifuging at 4,080 × g (5000 rpm, GSA) for 10 minutes at 4°C, resuspending, finally washed with 20 ml ice-cold 10% (v/v) glycerol and centrifuged as above. The pellet was resuspended in 4 ml ice-cold 10% (v/v) glycerol and the cells were stored at -80°C in 40 µl aliquots.

For transformation, the Gene Pulser (Bio-Rad) was set at 25 µF, 2.5 kV and 200 Ω. An aliquot (40 µl) of competent *E. coli* cells was mixed with 2 µl ligated DNA and

the mixture was transferred into an ice cold 0.2 cm cuvette then was pulsed at these settings. The cells were immediately suspended 1 ml of non-selective LB broth, incubated at 37°C for 1 hour and then plated at suitable dilutions onto selective LB Agar plates.

2.5.1.2 Preparation and transformation of $CaCl_2$ competent cells

To prepare competent *E. coli* cells, 1 L of LB broth was inoculated with 10 ml of overnight culture of *E. coli* strain, Top10 (or XL-1 blue), incubated at 37°C with vigorous shaking at 220 rpm to OD_{600} = 0.45-0.60. The cells were chilled on ice for 10 minutes and centrifuged at 5860 × g (6000 rpm, GSA) for 5 minutes at 4°C. The pelleted cells were resuspended in half the original volume of ice cold 0.1 M $CaCl_2$ and left on ice for 10 minutes. Cells were centrifuged again and resuspended again in 1/20 volumes in 0.1 M $CaCl_2$ and left on ice for at least 30 minutes before use. The competent cells were stored in17% glycerol at -80°C.

For transformation, 200 µl of competent *E. coli* cells were mixed with 2 µl ligated DNA and the mixture was left on ice for 40 minutes, subsequently it was heat shocked at 42°C for 2 minutes, placed on ice for 2 minutes, then 1ml non-selective LB broth was added. Following incubation at 37°C for 1 hour and plated on appropriate selective LB Agar plates the cells were incubated overnight at 37°C.

2.5.2 TRANSFORMATION OF *D. SEPTOSPORUM*

2.5.2.1 Preparation of competent *D. septosporum* protoplasts

Approximately 0.5 cm × 0.5 cm of *D. septosporum* mycelium was ground in 1 ml sterile Milli Q H_2O. The ground mycelia (100 µl) were inoculated into 25 ml low DB media in 125 ml flask. The flask was incubated at 22°C for 5 days, with shaking at 150 rpm. After 5 days, the mycelium was harvested by centrifuging at 5860 × g for 5 minutes in a GSA rotor. Then the mycelium was washed with sterile MilliQ H_2O for 3 times and with OM buffer for two times.

Approximately 30ml of filter sterilized (10 mg/ml) Glucanex (Novozymes Switzerland AG) was added to mycelia. The mixture was incubated at 30°C for 12-16

hours with shaking at 80-100 rpm. After incubation, the mixture was filtered through a sterile Mira cloth into a sterile flask. The protoplast mixture was divided into several 30 ml Corex tubes, 10 ml in each tube, and overlaid with 4 ml of ST buffer to a tube and centrifuged at 4°C for 5 minutes at 5860 × g (Rotor SS34). The protoplasts that formed a white layer at the interface of the two solutions were removed and washed 4 times with 5 ml STC buffer by centrifugation at 5860 × g (Rotor SS34) for 5 minutes at 4 °C. Finally the protoplast pellet was resuspended in 100 µl STC buffer and stored on ice.

The concentration of the protoplasts was determined by counting with a haemocytometer. Then the protoplasts were diluted to 1.5×10^8 protoplasts / ml with STC buffer.

2.5.2.2 Transformation of *D. septosporum* protoplasts

For each transformation, 80 µl of 1.5×10^8 protoplasts / ml in STC buffer were mixed with 5 µg DNA and incubated on ice for 30 minutes. Then 900 µl 40 % PEG 6000 solution were carefully mixed with the DNA/protoplast mixture and the final mixture was incubated for a further 20 minutes at room temperature. An aliquot of 100 µl final mixture was mixed with 5 ml DM top agar at 50°C and spread onto a DMSuc (10 ml) plate. The plates were incubated at 22°C overnight and overlaid with another 5 ml DM top agar that contained sufficient hygromycin B (Sigma) to make an overall concentration of 70 µg/ml. In each transformation, a sample of protoplasts alone was performed as a negative control (the procedure was exactly the same as the transformation samples). Aliquots (100 µl) of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions of the protoplast sample were grown on DMSuc plates without hygromycin B selection to count the viable protoplasts of the transformation. Aliquots (100 µl) of the negative control sample (protoplasts only) were spread on a DMSuc plate without hygromycin B selection and a DMSuc plate with hygromycin B selection to confirm the sensitivity of the protoplasts to hygromycin B.

Five µg pAN7-1 was used as a positive control, 4 aliquots (100 µl) were spread on DMSuc plates with hygromycin B selection to determine the transformation frequency.

2.6 AMPLIFICATION OF DNA BY THE POLYMERASE CHAIN REACTION (PCR)

2.6.1 Oligonucleotide primers

The primers were ordered from *Sigma Life Technologies* or *Invitrogen Life Technologies*. Each primer was re-suspended in sterilised MilliQ H_2O to a final concentration of 200 μM and stored at -20°C. The primers were diluted to a working stock concentration of 10 μM for PCR reactions and 3.2 μM for sequencing reactions. Table 2.2 lists the primers used in this study.

2.6.2 Reagents and Cycling Conditions for Basic PCR

The final concentrations of each component in 1 reaction were 1 × *Taq* polymerase buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 unit *taq* DNA polymerase (Invitrogen), and 0.5 μM of each primer and 1-3 ng of plasmid template or 3-10 ng of genomic template.

After mixing, the PCR reaction tubes were placed in a thermal cycler (Master cycler gradient; Eppendorf). The samples were then subjected to an initial denaturation step of 2 minutes at 94°C, then 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds (adjusted depending on primers used and specificity wanted), extension at 72°C for 1 kb/minute (depending on expected product size), and with a final 10 minutes extension at 72 °C.

2.6.3 *E. coli* colony PCR

E. coli colony PCR was used to screen *E. coli* transformants. The colony was used as a template. A sterilised P10 or P200 pipette tip was used to pick up the colony cells, a gentle touch at the colony edge is enough for a PCR reaction. The cells were suspended in the reaction mixture used for a basic PCR. The components are same as the basic PCR and the cells kept in the reaction system, so too many cells might inhibit the PCR reaction. The PCR program was 1 cycle of 3 minutes at 96°C to expose the cell components, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds (adjusted depending on primers used and specificity wanted), extension at 72°C for 1 kb/minute (depending on expected product size), and 1 cycle of 5 minutes at 72°C.

Table 2.2 Sequencing and PCR primers

Primer	Size (nt)	Tm * (°C)	Sequence (5'to 3')	Source /reference
pUC/M13 Foward	22	70	GCCAGGGTTTTCCCAGTCACGA	Perkin Elmer
pUC/M13 Reverse	24	70	GAGCGGATAACAATTCACACAGG	Perkin Elmer
SP6	22	58	TTTAGGTGACACTATAGAATAC	Promega
T7	23	66	TAATACGACTCACTATAGGGCGA	Promega
GeneRacer 5'primer	23	74	CGACTGGAGCACGAGGACACTGA	GeneRacer kit
GeneRacer 5' nested primer	26	78	GGACACTGACATGGACTGAAGGAGTA	GeneRacer kit
GeneRacer 3'primer	25	76	GCTGTCAACGATACGCTACGTAACG	GeneRacer kit
GeneRacer 3'nested primer	23	72	CGCTACGTAACGGCATGACAGTG	GeneRacer kit
5'dotA RACE	24	76	GTTGACACGGAAGACGCGGTCGAA	This study
5'dotB RACE	24	76	GATCTGCGCATCGGAGGTGTATCC	This study
5'dotC RACE	24	76	TCCTCCCGTGCGTGAGCATCTTCA	This study
5'dotD RACE	23	74	TAGACGGCGAGGTCGCGAGAGAT	This study
avf5' RACE	23	76	GACGGTGTCGGCGGTGAGAGAGA	This study
cyp5'RACE	21	78	GCATCGCCCTCGCCCTCCTGC	This study
epo5'RACE	22	78	CCGATGGGGGAGAGGCGGATCA	This study
5'epoRACE	24	78	TCCCAGATCGCCGCCTTGGGCAAT	This study
mox5' RACE	22	72	GGGGGCGTTCCAGGTCGTCTTT	This study
5'pks RACE	26	80	GCCGGCACATACTTCTTCACAGCAT	This study
pks 5' RACE	23	78	GCGGCAAGCGAGCCGGTGCAGAT	This study
dot A 3' RACE	24	76	GCACGCCATCTACTCTGGCTCCAA	This study
dot B 3' RACE	25	76	CGAAGCCAACACGACCAATCTCCAA	This study
dot C 3' RACE	24	72	TGCTCGTCAGCAAGACTGAGTTGA	This study
dot D 3' RACE	25	72	CGAAGCCAACACGACCAATCTCCAA	This study
avf3' RACE	23	76	GTGGGGAGTGCTGGCGGGATTCT	This study
cyp3' RACE	23	76	GGCGAGGGCGATGCTGCGACGTT	This study
epox3'RACE	23	74	ACCCCTACCGTCGCTTCGCTTCT	This study
mox3' RACE	23	74	CGGGAGTCTGGTTCGGGGTGATA	This study
pks3' RACE	22	74	CTTACGCCCGCGACCCCGAGAA	This study
3'epoA	20	58	TGGCAGCGTTAAAAAGGCTT	This study
3'avfA	23	60	CATCTTGAAATTCGAAGTTTGA	This study
5' mox-out	20	60	CTGCTCAGCGTCATGGGAAA	This study
5'epox-out	18	56	TCA CAG CCT TCC GAA ACC	This study
5'epox-in	18	58	GAG CAC TTT AGC CCG AGC	This study
5'ord-in1	21	62	ACTTGACATTTCCAGCCGTCT	This study
5'ord-in2	20	62	GACACTGACGTGGTAGGGTC	This study
5'cyp-in1	18	50	TCAACGGATTGCATGTAA	This study
5'cyp-in2	23	66	ACTTACTGTTCTTGACGCCG	This study
5'pks-in	22	70	TCTCGCAGGCAAGACCCGAAGA	This study

Primer	Size	Tm * (°C)	Sequence (5'to 3')	Source /reference
epoAKO1	52	154	**GGGGACAACTTTGTATAGAAAAGTTG** GGTGATGTGTGGTAACGAGCGTTGC	This study
epoAKO2	52	154	**GGGGACTGCTTTTTTGTACAAACTTG** CAGGTCTTGGAGGGTTTGGAGTTTGG	This study
epoAKO3	51	156	**GGGGACAGCTTTCTTGTACAAAGTGG** CTTGGAGACCGTCTCCCTATACTGG	This study
epoAKO4	52	154	**GGGGACACCTTTGTATAATAAAGTTG** CTCCTCGTTCGACGATTGAACGGCAG	This study
5'hphout	22	68	GAATCTCCGGTGTGGAAGA	Teddy 2004
3'hphout	23	68	TCCTTGAACTCTCAAGCCTACAG	Teddy 2004
pksCA	20	63	CTT TCC TCG CTG TCT TCC TC	Seconi 2001
dmoF2	18	58	GTCTTCGATGACGCGGAGG	Morgan 1997
R163overlap	20	62	TTCCCAGAGGCACAAAGCAC	Seconi 2001
Dpks1	19	62	CTCGTTGATTATACCCTTCTCC	Morgan 1997
Dpks2	22	64	GGAGAAGGGTATAATCAACGAG	Morgan 1997
8reverse6	20	62	GCAAGAGGGAATGCTGTCAA	Jin 2005
R162reverse1	20	64	TTCTCGACAACAATCGTCCATA	Teddy 2004
R162reverse2	21	64	GTGTGGTAACGAGCGTTGCAA	Teddy 2004
R162reverse5	24	70	GCAGACATGAGAGTAAGTCCAGTT	Teddy 2004
R162forward2	23	68	CAAGATGACTCTCGGAGTTTCAG	Teddy 2004

* Melting temperature calculated as **Tm (°C)= 2 (A+T) +4 (G+C)** from Itakura *et al* (Itakura *et al.*, 1984).

Underlined bold region in the primers *epoAKO1*, *epoAKO2*, *epoAKO3* and *epoAKO4* are *attB* sites for the gateway recombination.

2.7 DNA SEQUENCING

All plasmids sent for sequencing at the Institute of Molecular BioScience (IMBS)/ Allan Wilson Centre Genome Analysis Centre were purified by a commercial plasmid purification kit and concentrated by Flurometer (Section 2.3.8). Plasmid DNA (200 ng) or PCR product (2 ng/100bp), 3.2 pmol / µl primer was used for sequencing.

2.8 SOUTHERN BLOTTING AND HYBRIDISATION

2.8.1 Southern Blotting (Capillary)

The DNA blotting method used was based on that of Southern (Southern, 1975). DNA for Southern blotting was prepared using the CTAB method (Section 2.3.2). It was digested as in Section 2.4.1, and separated by gel electrophoresis (Section 2.3.7), through a 0.7% agarose gel, run at low voltage (1-2 volts/cm) overnight at 4°C, stained in ethidium bromide and photographed with a ruler alongside the gel. The gel was gently agitated in 250 mM HCl for 15 minutes to depurinate the DNA so that high molecular weight regions would transfer efficiently. The DNA in the gel was then denatured by gentle agitation in denaturing solution for 30 minutes. The gel was neutralised by washing twice in neutralising solution for 20 minutes per wash. Before placing on the blotting apparatus, the gel was soaked in 20 × SSC for 30 minutes. The blotting apparatus was constructed as described in Molecular cloning (Sambrook and Russell, 2001). After blotting overnight, the apparatus was disassembled and the Hybond-N^+ membrane (Amersham) was treated with shortwave UV light for 3 minutes to crosslink the DNA to the membrane then air-dried.

2.8.2 DIG labelling of DNA probe

DNA probes were labelled with Digoxygenin (DIG)-11-dUTP with a DIG-random priming labelling kit (Roche) according to the manufacturers' instructions.

2.8.3 Southern Blot Hybridisation of DIG Labelled Probe

This was carried out according to principally the DIG High Prime DNA Labeling and detection starter Kit II instruction Manual; Roche.

The membrane to be probed was placed in a hybridisation tube and 10 ml of DIG Easy Hyb solution (Roche) (10 ml/100 cm^2 of membrane) was added to the tube. The membrane was placed in a rotary oven at 42°C and prehybridised for one hour. The labelled probe was denatured by heating for 5 minutes in a boiling water bath and plunging immediately into ice. After prehybridisation, the prehybridisation solution was replaced with fresh DIG Easy Hyb solution that contained 2.5 ng of the denatured probe per ml of DIG Easy Hyb. The membrane was placed in a rotary oven at 42°C and hybridised overnight.

After hybridisation, excess probe was washed off the blot as follows. The membrane was removed from the hybridisation tube with forceps, and washed twice in 200 ml of Wash solution I (15-25°C) on a shaking platform for 5 minutes. Then the membrane was washed twice with 200 ml of pre-warmed Wash solution II and incubated on a shaking platform at 68°C for 15 minutes with constant shaking.

2.8.4 Immunological Detection

The membrane was washed for 1-5 minutes in Washing buffer. The membrane was then incubated in Blocking solution for 30 minutes and then incubated in Antibody solution for 30 minutes. The membrane was washed twice for 15 minutes in washing buffer and then equilibrated for 1-5 minutes in Detection buffer. The wet membrane was placed onto acetate paper with the DNA side facing up. One ml CSPD ready-to-use lumigen (Roche) was dispensed over the surface of the membrane and a second acetate sheet was placed over top. The CSPD lumigen was spread evenly and gently over the surface and left for 5 minutes at 15-25 °C. The excess liquid was squeezed out, the edge sealed and the membrane incubated at 37°C for 15 minutes. The membrane was then exposed to X-ray film (Kodak Scientific Imaging) for 1 hour. The X-ray film was developed by an automated developer (100 Plus Automatic X-ray Processor, All Pro Imagining).

2.8.5 Stripping

The membrane was stripped by washing thoroughly in double distilled water, then washing twice in Stripping buffer at 37°C for 15 minutes. Then the membrane was rinsed thoroughly in 2 × SSC for 5 minutes. The stripping was confirmed by immunological detection. The stripped membrane could be stored in 2 × SSC until used again.

2.9 **RACE** (Rapid Amplification of cDNA Ends)

A GeneRacer™ Kit (Invitrogen) was used to identify the 5'and 3'untranslated regions of the genes. The method involves a dephosphorylation reaction with calf internal phosphatase (CIP) to remove the 5'phosphates, followed by a de-capping reaction with tobacco acid pyrophosphatase (TAP) to remove the 5'cap structure from the full-length mRNA. Subsequently the GeneRacer™ RNA Oligos are ligated to the 5'end of the mRNA with T4 RNA ligase. The ligated mRNA was reverse transcribed with SuperScript™ III RT and the GeneRacer™ Oligo dT primer (or Random primer for big genes such as the *pksA* gene) to create RACE-ready first-strand cDNA. Each reaction involved in the RACE was carried out according to the manufacturers' instruction. Seven μg total RNA was used at the beginning. After each reaction the mixture was cleaned up by QIAGEN PCR column, including the first-strand cDNA.

5'RACE was performed by using the GeneRacer™ 5'primer (complementary to the ligated oligo region) and reverse gene-specific primer (GSP), which was designed according to the guidelines of the manufacturer (Tm ≥ 72 °C, n>23). 3'RACE was the same as 5'RACE, except using one primer from the GeneRacer™ oligo dTs (the GeneRacer™ 3'primer) and another primer designed to be a forward gene specific primer (GSP). The amplification reaction was carried out according to the manufacturers' instruction. Platinum® *Taq* DNA polymerase High Fidelity (Invitrogen) was used for RACE amplification. The cycling conditions were 1 cycle of 94°C for 2 minutes; 5 cycles of 94°C for 30 seconds and 72°C for 1 kb/minute; 5 cycles of 94°C for 30 seconds and 70°C for 1 kb/minute; 25 cycles of 94°C for 30 seconds, 65°C for 30 seconds and 72°C for 1 kb/minute; 1 cycle of 72 °C for 10 minutes.

Nested PCR was also used to amplify RACE products. The reagents used were the same as for basic PCR (Section 2.8.2). The program was 94°C, 2 minutes; 94°C, 30 seconds, 65°C, 30 seconds, 72°C, 1 kb/minute, 25 cycles; 72 °C, 10 minutes. The nested PCR products were purified by gel purification (Section 2.3.4) or PCR column (Section 2.3.5) and cloned into pGEM-T or pGEM-T Easy vector and sequenced (Section 2.7). The sequences of RACE PCR products were aligned with genomic DNA sequences to identify the 5' and 3'ends of the targeted genes with software Gene Jockey II.

2.10 RNA MANIPULATION PROCEDURES

2.10.1 Isolation of total RNA

The method used for extraction RNA was modified from the Micro to Midi total RNA purification System (Invitrogen). Approximately 250 mg fresh fungal mycelia (frozen in liquid nitrogen) were ground to a fine powder in liquid nitrogen, and mixed with 1 ml of RNA lysis solution (Micro to Midi total RNA purification System kit) containing 10 µl of 2-mercaptoethanol. The mixture was left for 3 minutes at room temperature, then homogenized with gentle pipetting, followed by centrifugation at 12,000 × g for 2 minutes at 25°C. Two volumes of 100% ethanol were added to precipitate the RNA. The mixture was left for 10 minutes on dry ice (or -80°C), centrifuged at 15,700 × g for 10 minutes at 4°C and the supernatant discarded. The RNA pellet was washed with 1 ml of 70 % ethanol, air-dried, and re-suspended in 100 µl DEPC-treated water.

2.10.2 DNase treatment of RNA

RNA samples were treated with TURBO DNase™ (RNase free) (Ambion, Inc., Austin, TX) to remove residual DNA. Total RNA (1 µg) was incubated with 1 unit TURBO DNase™, in 1 x DNase buffer and 1 × RNA secure (an RNase inhibitor) (both buffer supplied with DNase) at 37°C for 1 hour. The mixture was then extracted with 2 volumes of chloroform and centrifuged at 15,700 × g for 10 minutes. The RNA was precipitated with 1/10 volume of 3 M sodium acetate and 2 volumes of 100 % (v/v) ethanol, stand at -80 °C for 10 minutes, and recovered by centrifuging at 15,700 × g for 10 minutes. The pellet was washed in 70% ethanol, air dried and resuspended in DEPC-H_2O and kept at -20°C (short term) or -80°C (long- term).

2.10.3 Quantification of RNA

RNA was quantified by measuring the absorbance at 260 and 280 nm using an Ultrospec 3000 UV/visible spectrophotometer (Pharmacia Biotech). Samples were usually diluted so that the absorbance reading was between 0.1 and 1.0. Good quality RNA samples should have a ratio of A260/A280 greater than 1.8. The concentration of RNA was calculated by the spectrophotometer as *n* µg/ml, and the dilution factor taken into account.

2.10.4 Reverse transcription and RT-PCR

Two methods were employed for amplifying cDNA. Reverse transcription can be used to obtain cDNA that can be used as template for normal PCR; it is relatively cheap compared to one step PCR, but the first strand cDNA is relatively unstable. If there are several RT-PCR reactions from the same strain to be done during a short time period, such as using RT-PCR to check RACE results, reverse transcription is suitable. One step RT-PCR is very convenient for amplifying cDNA of individual genes from different templates, such as checking differences in gene expression between the wild type and mutants.

2.10.4.1 Reverse transcription

Transcriptor First Stand cDNA Synthesis kit (Roche) was used for transcription of the first strand cDNA. Total RNA (3 μg) was used for the reaction. The reaction followed that of the kit instruction manual, except that the reverse transcription reaction was performed at 50°C for 1 hour. The first strand cDNA (0.5-1.0 μl of the transcription reaction) was directly used as template for normal PCR (Section 2.6.2).

2.10.4.2 RT-PCR

RT-PCR was performed by using SuperScript™ One-Step RT-PCR kit (Invitrogen) according to the instructions of the manufacturer. Total RNA (100 ng) was used as a template for reverse transcription, incubated at 50°C for one hour, followed by the general PCR program (Section 2.6.2)

2.10.5 AGAROSE GEL ELECTROPHORESIS OF RNA

2.10.5.1 SDS / Agarose Gel electrophoresis of RNA

Total RNA was checked for 28s and 18s rRNA bands by electrophoresis through a 1.5% agarose gel (containing 0.3% SDS) disolved in 1 × TBE buffer at 5-8 volts/cm. Dyes (bromophenol) present in the gel loading buffer, which was mixed with RNA samples before loading, allowed estimation of RNA migration. When the blue dye shifted to ~2.0 cm from the edge of the gel, then the gel was ready. After

electrophoresis, agarose gels were stained in ethidium bromide for 10-20 minutes, washed in water, observed under short wave UV light (254 nm) and photographed.

2.10.5.2 Formaldehyde Gel electrophoresis of RNA

A 1.2% formaldehyde gel (150 ml) was made with 1.8 g agarose melted in 110 ml DEPC treated H_2O. After cooling to 60°C, 15 ml 10 × MOPS buffer and 25 ml 37 % formaldehyde were added. RNA sample (4.7 µl) was mixed with a master mix (cocktail) that contained (per sample) 2 µl 10 × MOPS buffer, 10 µl formamide, 3.2 µl (37%) formaldehyde and 0.08 µl ethidium bromide, this mixture was incubated at 65°C for 15 minutes to denature RNA. Two µl BPB loading dye (Appendix A2.5) was added to each RNA sample before the samples were loaded on the gel. The gel was run in 1 × MOPS buffer at 5-8 volts / cm (1-2 volts/cm for Northern Blot). When the dye (bromophenol) shifted to ~2.0 cm from the end of the gel, then the gel was ready and observed directly under short wave UV light (254 nm) and photographed.

2.11 NORTHERN BLOTTING AND HYBRIDISATION

2.11.1 Northern blotting

The RNA samples for northern blotting were run on a 1.2% formaldehyde agarose gel at low voltage (1-2 volts/cm) overnight in the fume hood. The gel was photographed with a ruler alongside the gel. The blotting apparatus was constructed as for Southern blotting (Section 2.8.1), except that 20 × SSC was treated with DEPC. After blotting overnight, the apparatus was disassembled and the Hybond-N^+ membrane (Amersham) was exposed to shortwave UV light for 3 minutes to crosslink the RNA to the membrane, then air-dried in the dark.

2.11.2 Probe labelling

Probe DNA was radio-labelled with [α-^{32}P] dCTP using a High Prime Labelling Kit (Roche). DNA (100 ng) in a volume of 11 µl was denatured in a boiling water bath for 10 min and immediately placed on ice. To this 4 µl of High Prime Solution and 5 µl (50 µCi) [α-^{32}P] dCTP (Amersham) was added. The reaction was mixed, spun briefly in a

centrifuge and incubated at 37°C for 1 hour. The reaction was stopped by addition of 2 µl of 0.2 M Na_2EDTA (pH 8.0) and 28 µl TES buffer (10 mM Tris-HCl, 0.1 mM EDTA, 100 mM NaCl).

2.11.3 Hybridisation

For hybridisation, the nylon membrane (Amersham) was pre-hybridised with 30 ml of hybridization solution at 65°C for at least 2 hour. After pre-hybridisation, boiled radio-labelled probe was added to the tube and hybridisation was carried out at 65°C overnight. The membrane was removed and pre-washed in Wash solution I for 20 minutes at room temperature and in Wash solution II for 20 minutes three times at 68°C. The washed membrane was exposed to X-ray film (Kodak Scientific Imaging) at –80°C for overnight. The X-ray film was developed by an automated developer (100 Plus Automatic X-ray Processor, All Pro Imaging).

2.12 ISOLATION OF SECONDARY METABOLITES FROM MYCELIUM OF *D. SEPTOSPORUM*

Five agar plugs (diameter 5mm) of mycelia from a 7 day culture grown on a DM plate were ground in 1 ml sterilized MilliQ H_2O. Then 200 µl of the suspension was inoculated into each of four 2 L flasks containing 200 ml low DB media. The flasks were incubated at 22°C for 9 days with shaking (~100 rpm).

The contents of all flasks were collected into one flask. Half a volume (400 ml) of acetone was added, swirled and left to stand for 30 minutes. Half a volume (400 ml) of chloroform was then added and the contents of the flask were shaken thoroughly. The contents were filtered through a nappy liner into a separatory flask and the contents were left to settle out into two layers. The bottom (organic) layer was separated into a round flask and placed on a rotary condenser under vacuum until approximate 5 ml of chloroform was left. Then the last 5 ml of the chloroform/polyketide solution was transferred to a small round flask and left in a fume hood to evaporate the last of the chloroform. One ml of chloroform was then added to the small round flask and swirled until all of the polyketide was dissolved. This mixture was then transferred into a glass vial and left in a fume hood (2-4 days) to evaporate the last of the chloroform. Then the

glass vial was placed over a glass Petri dish of phosphorus pentoxide in a vacuum sealed desiccator (which was left within a cage in case of implosion) for 2-3 days in order to get rid of the residual water.

2.13 DETECTION OF AFLATOXIN INTERMEDIATES

Metabolites prepared in Section 2.12 were sent to the Southern Regional Research Centre, United States Department of Agriculture (SRRC USDA), New Orleans, U.S.A. to be tested for the presence of aflatoxins. Samples were analysed using thin-layer chromatography (TLC) by resuspending samples in chloroform, spotting suspension on reverse phase TLC plates and comparison to aflatoxin intermediate standards. Plates were run in ether-methanol-water solvent systems. The position of the spot on the plate was verified by running extracts in several solvent systems.

2.14 INTERMEDIATE FEEDING STUDY

One 5 mm diameter plug of mycelia was taken from 7 day old plates of mutants P6 and P53, ground with a plastic pestle and inoculated into 10 ml low DB media in a 125 ml flask containing 20 μg/ml intermediates (versicolorin A, norsolorinic acid, averufin or averythrin) and incubated at 22°C with constant shaking at 160 rpm, for 7-10 days. After incubation, the intermediates were extracted as in Section 2.12. A 5 ml (half volume) acetone was added to the media and mycelia, swirled and left for 30 minutes. Then 5 ml of chloroform was added to the mixture, swirled and left for another 30 minutes. Finally the mixture was transferred into a 15 ml sterilized chloroform resistant tube and centrifuged at 3220 × g for 10 minutes. The bottom phase (chloroform) was removed by a pipette into a 20 ml glass bottle, left in the fume hood for 36 hours and then the residue was transferred into a microcentrifuge tube and left in the hood until dry. Then the intermediates were dissolved in 10 μl MeOH containing 0.1% formic acid and checked on a TLC plate, which was run in MeOH-H_2O (2:1) contain 4% formic acid solvent system (Franich, 1981). Mutant without intermediates and intermediates without mycelium were used as negative controls, wild type extract was used as positive control.

2.15 QUANTIFICATION OF DOTHISTROMIN USING COMPETITVE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA).

Dothistromin levels were measured using the competitive ELISA method described by Jones *et al* (Jones *et al.*, 1993). In this method, dothistromin (Doth) was fixed on the microtitre plate first. Then the mixture of the samples and a peroxidase labelled monoclonal antibody (per 10C12) was added to the plate. The dothistromin on the plate would compete with the dothistromin in the samples to interact with antibody. A special substrate mixture was used to detect the antibody that remained bond to the plate after washing. The more the antibody binds to the plate, the less dothistromin was present in the sample obtained and the darker colour from the reaction. So, negative control (blank samples) will have the darkest colour (the greatest absorbance) amongst the tested samples. Similarly a lower absorbance means there is more dothistromin in the sample. ELISA was performed as follows.

Microtitre wells were coated (100 µl/well) with Doth-MSA (5 µl) conjugate (kindly provided by Dr. Bill Jones, HortResearch) diluted in phosphate-buffered saline (PBS 15 ml). The plates were covered and incubated at 37°C for 3 hours. The plates were then washed five times with PBS plus 0.1% Tween-20 (PBST) before 1% skimmed milk power in PBS plus 1% thiomersal was used to block the remaining protein-binding sites on the microwell surface (400 µl/well). The plates were stored at 4°C and washed once with PBST before use.

Standard solutions were prepared by diluting stock dothistromin solution in DMSO. Working standards were then prepared by diluting 1 µl of the standard solutions in 1 ml of modified dilution buffer containing equal volumes of 2 × PBST (+ 2% skimmed milk powder) and low DB medium.

Macerated mycelium was used to inoculate 25 ml of low DB and incubated at 22°C while shaking for 7 days. Samples (200 µl) were removed from the flasks and centrifuged at 15,700 × g for 5 minutes in a microcentrifuge to remove any mycelial fragments and spores. Liquid broth samples were then prepared by adding 150 µl of sample to 150 µl of working buffer (2 × PBST, 2% skimmed milk powder, 0.2%

DMSO). DMSO was added to standardise the samples and the working standards. Further dilutions of the liquid samples were performed in modified working buffer which contained equal volumes of working buffer and low DB media.

Standards and samples (100 µl of each) were pre-incubated at 37°C for 1 hour with 100 µl of peroxidase-labelled monoclonal antibody, per 10C12 (1:30000 in dilution buffer). Aliquots (100 µl) were then transferred to the washed Doth-MSA microtitre plates and incubated at 37°C for 3 hours. After the incubation, plates were washed six times in PBST to remove any free labelled peroxidase. freshly prepared substrate mixture (200 µl) (40 mg *o*-phenylene diamine, 0.51 g citric acid, 40 µl of 30% H_2O_2, 27.7 ml 0.2 M Na_2HPO_4, made to a total volume of 100 ml with water) was added to each Doth-MSA microtitre well. The plates were covered in tinfoil and shaken at room temperature for 30 minutes. The peroxidase reaction was stopped by the addition of 50 µl of 4 M sulphuric acid to each well. Absorbance was measured at 490 nm with reference at 595 nm in a dynatech MR 5000 plate reader and the percentage inhibition for each sample and standard was calculated. A standard curve of percentage inhibition against dothistromin concentration was constructed and unknown concentration determined.

2.16 GROWTH RATE ANALYSIS

Three mm diameter agar plugs from a 7 day old culture were used to inoculate the growth test plates. DM plates were used for the growth test. There were three colonies on each plate, one of wild type, one of an ectopic transformant and one mutant. Nine replicates were prepared for each strain and the plates were incubated at 22°C for four weeks. The diameter of each colony was measured along two axes intersecting at right angles. The average of these two measurements was taken as the diameter of the colony on each plate.

2.17 EXAMINATION OF CONIDIA FORMATION

Five mm diameter agar plugs from a 7 day old culture were used to inoculate the test plates. The test strains were inoculated onto DSM plates, 4 or 5 colonies on each plate, including one wild type, one ectopic and at least one mutant. Five replicates for each

sample were prepared and incubated at 22°C for 3 weeks. The conidia can usually be observed at the margins of a colony. Two plugs of mycelia (5 mm diameter) were taken from the margins on opposite sites of each colony, placed into 200 µl 0.1% Tween-20 (diluted in sterilized MilliQ H_2O) and vortexed for one minute. Then 5 µl 0.1% Tween-20 solution of each sample was counted with a haemocytometer.

RESULTS & DISCUSSIONS

CHAPTER 3: *PKSA* GENE DISRUPTION

CHAPTER 4: *EPOA* GENE DISRUPTION

CHAPTER 5: RACE RESULTS

CHAPTER THREE: *PKSA* GENE DISRUPTION

3.1 CHARACTERIZATION OF THE PUTATIVE DOTHISTROMIN GENE, *PKSA*

The putative *D. septosporum pksA* gene has been proposed as a key dothistromin gene on the basis of amino acid sequence similarity to PksA in the AF/ST cluster. Dothistromin is a polyketide and a secondary metabolite of *D. septosporum*. AF and ST are also polyketides, and an early step in polyketide synthesis is catalyzed by the enzyme polyketide synthase. AF and ST biosynthesis (Fig 1.2) start with conversion of malonylCoA to hexanoylCoA by two fatty acid synthases and then polyketide synthase uses the hexanoyl-CoA as a starter unit to assemble the carbon chain (Yu and Leonard, 1995). If *D. septosporum pksA* has a similar function to its homolog in the AF/ST cluster, targeted disruption of the *pksA* gene will prevent assembly of a functional polyketide synthase and will block the dothistromin biosynthetic pathway at a very early step. There should be no other secondary metabolites accumulated as intermediates by the mutants.

The putative *pksA* gene contains six conserved domains: ketoacyl synthase (KS), acyl transferase (AT), three acyl carrier proteins (ACP) and one thioesterase (TE). The *pksA* gene disruption vector was designed to replace two conserved domains, KS and AT, with a selectable *hph* gene cassette. The *pksA* disruption vector pR226 was constructed by Teddy (2004). The targeted gene replacement of *pksA* still requires transformation of *D. septosporum*, screening and confirmation of the targeted disruptants, characterization of *pksA* mutant strains by checking for dothistromin biosynthesis and intermediate feeding studies to confirm the gene function.

3.2 TRANSFORMATION OF *D. SEPTOSPORUM*

The transformation of pR226 was carried out with a transformation system that has been developed and used for characterizing the function of the *dotA* gene. Only one strain of *D. septosporum* has been identified in New Zealand (Hirst *et al.*, 1999), but different isolates are used for research. The latest isolates of *D. septosporum*, NZE7 and NZE8 were used for the transformation due to their high dothistromin expression. The protoplast quality for both NZE7 and NZE8 was estimated by their regeneration efficiency, which was obtained by plating a serial dilution of protoplasts (treated the

same but without DNA) onto non-selective plates (Section 2.5.2.2). On basis of this, it was estimated that 1% of protoplasts were able to regenerate. Undiluted protoplasts were also plated on selective plates as a negative control. There was no colony growth on the plates. The vector pAN7-1 (containing hygromycin B resistant gene, *hph*) was used as a positive transformation control, there were 424 colonies generated on the selective plates. The transformation efficiency was calculated as transformants per μg DNA. The transformation results are shown in Table 3.1. Transformations of NZE7 and NZE8 with pAN7-1 and the *pksA* disruption plasmid pR226 were performed at the same time (rows 1-3 in Table 3.1). Transformation of NZE8 was repeated (row 4) since there were only 4 transformants at the first time (Table 3.1).

Table 3.1 Transformation results of pR226

	Plasmid (5 μg)	*D. septosporum*	Total Transformants (5 μg)	*Transformation efficiency
1	pAN7-1 (1 μg/ μl)	NZE7	424	84.8
2	pR226 (125 ng/μl)	NZE7	50	10
3	pR226 (70 ng/μl) First time	NZE8	4	0.8
4	pR226 (221 ng/μl) Second time	NZE8	87	17.4

*** Transformation efficiency was calculated as transformants per μg DNA**

As shown in Table 3.1, different vector concentrations have different transformation efficiency. Especially, for the first time transformation of NZE8, 5 μg 70 ng/μl vector pR226 DNA only had 4 transformants, but the second time, 5 μg of 221 ng/μl vector DNA had 87 transformants. Five μg of 1 μg/ μl positive control vector pAN7-1 had 424 transformants of NZE7 transformation. Five μg of 221 ng/ μl vector pR226 had 50 transformants of NZE7 transformation. The regeneration efficiency of the protoplasts in NZE7 and twice NZE8 transformation were all estimated as 1%. The transformation procedures were the same. The differences in transformation frequency suggested that the vector DNA concentration had an effect on the transformation efficiency: the higher DNA concentration the greater transformation efficiency.

After the transformed protoplasts had been incubated at 22°C for a week, tiny colonies could be observed on the regeneration plates. Once the transformants grew to 3-5 mm (about 2 weeks), then they were subcultured to new DM hygromycin B selective plates

(normally 10 colonies on each plate). After one week's growth on the selective plates at 22°C, some transformants showed hygromycin resistance but some of them could not grow. Subsequently mycelia of the hygromycin B resistant colonies were transferred to a new DM hygromycin plate (4 colonies on each plate). After 7-10 days growth, ~ 0.5 cm^2 mycelia of each colony were used for preparing DNA (Section 2.3.1) and preliminary screening by PCR (Section 2.6.2).

In total, 141 hygromycin B resistant transformants were transferred from all the pR226 transformation. After growth on a DM selective plate for a week, some transformants showed a very pale color. Those pale colonies along with some normal looking colonies were subjected to a preliminary screen by PCR. The pale transformants were shown to be potential *pksA* mutants.

3.3 IDENTIFICATION OF THE *PKSA* GENE DISRUPTED MUTANTS

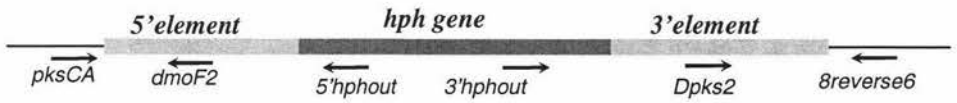
3.3.1 PCR to screen the transformants

The 5'and 3' elements of the *pksA* gene disruption vector pR226 are regions of the *pksA* gene designed for homologous recombination. Two conserved domains of the *pksA* gene, KS and AT, should be replaced by the *hph* gene. The genomic DNA of pR226 transformants was extracted (Section 2.3.1) and screened by PCR (Section 2.6.2) using the primers shown in Fig 3.1a.

The primer *pksCA*, upstream of the 5'element region, and *5'hphout*, a primer at the 5'end of the *hph* gene region, were used to screen for targeted integration at the 5'element end; *8 reverse6*, a primer downstream of the 3' element region and *3'hphout*, a primer at the 3'end of *hgh* gene region, were used to screen for integration at the 3'element end. Primers, *dmoF2* in the 5'element and *Dpks2* in the 3'element were used as positive controls for each PCR reaction, respectively. The 5'region PCR should give a 2.0-kb product and the 3'region PCR should give a 1.1-kb product. The positive control primers should give 0.85-kb and 0.65-kb PCR products from the 5'and 3' regions respectively. If both 5'and 3' region screening primers gave PCR products, the targeted region of the mutant was replaced by the *hph* gene and a double crossover had occurred in the mutant.

Fig 3.1 PCR screening the *pksA* gene disruptants

a. Schematic map of PCR to screen the *pksA* gene disruptants



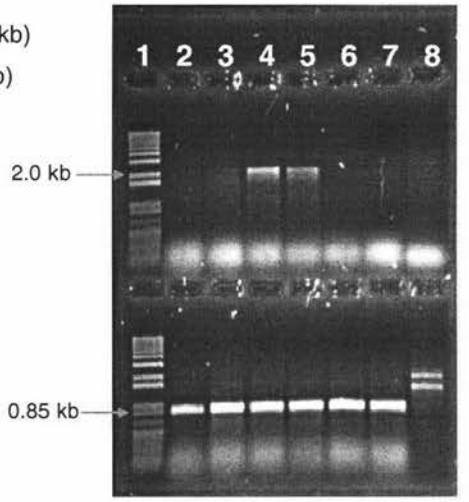
→ Primers, with names and positions indicated. The primer pair, *pksCA* and *5'hphout* were used to screen the 5'end integration; the primer *dmoF2* and primer *pksCA* were used as PCR positive control; the primer pair *3'hphout* and *8reverse6* were used to check the 3'end integration and the primer pair *Dpks2* and *8reverse6* were used as positive control.

— The *pksA* gene (flanking the 5'and 3'elements).

b. PCR to check 5'end of pksA/NZE7 knockout

Mutant sceen (Top): *pksCA* + *5'hphout* (2.0kb)
Controls (Bottom): *pksCA* + *dmoF2* (0.85 kb)

Lane 1 1 kb $^{+}$ ladder
Lane 2 P16
Lane 3 P12
Lane 4 P8
Lane 5 P6
Lane 6 P4
Lane 7 P1-7
Lane 8 WT/ NZE5



c. PCR to check 3'end of *pksA*/NZE7 knockout

Mutant screen (Top): *8reverse6* + *3'hph out* (1.1 kb)
Controls (Bottom): *8reverse6*+ *Dpks2* (0.65 kb)

Lane 1 1 kb $^{+}$ ladder
Lane 2 P16
Lane 3 P12
Lane 4 P8
Lane 5 P6
Lane 6 P4
Lane 7 P1-7
Lane 8 WT/ NZE5

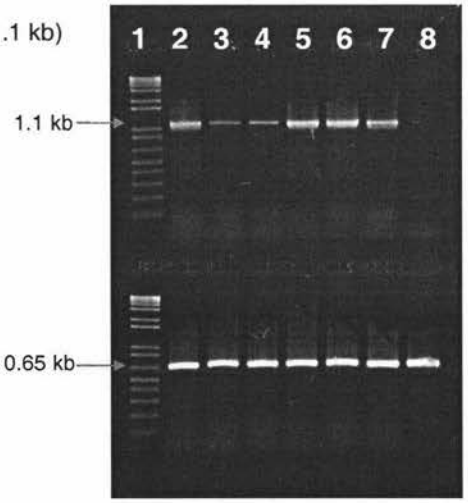


Fig 3.1b and c shows the PCR results of the possible *pksA* disruptant colonies P1-7, P4, P6, P8, P12 and P16. P8 and P6 gave a clear 2.0-kb band from 5' region PCR (Fig 3.1b. top gel lane 4 and 5) and a clear 1.1-kb band from 3'region PCR (Fig 3.1c, top gel, lane 4 and 5), as expected for a targeted gene replacement. Colony P12 (Fig 3.1b top gel lane 3) also had a very weak 2.0-kb band from 5'element PCR. A double cross-over event might have occurred in these three colonies. The other colonies, P16, P4 and P1-7, only had PCR products from the 3'region PCR (Fig 3.1c. lanes 2, 6 and 7); but not from the 5' region PCR (Fig 3.1b. lane 2, 6 and 7). A single crossover integration event in the 3' region might have occurred in these colonies.

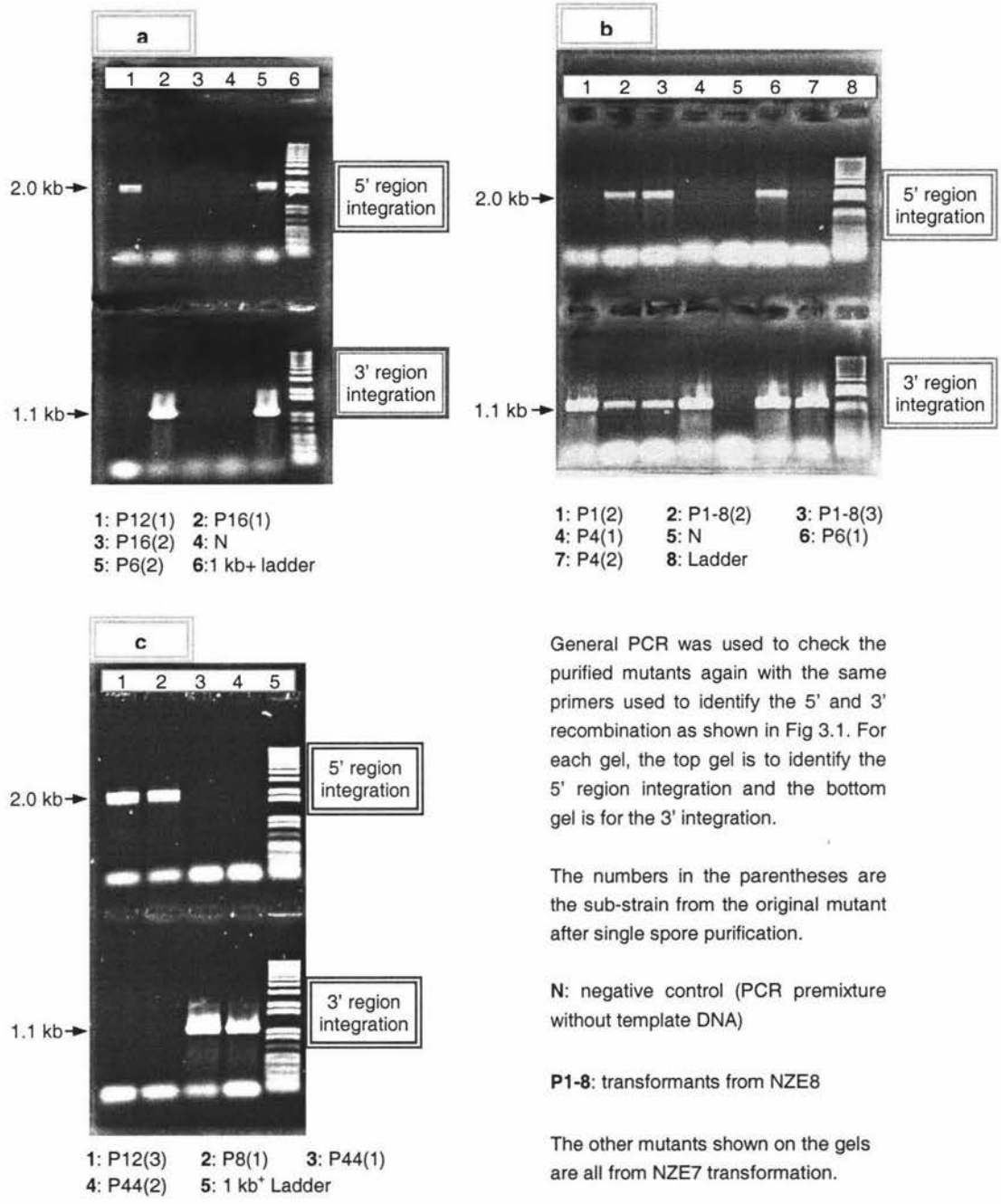
Wild type (WT) NZE5 DNA was used as template for the control PCR and used as a negative control for the 5'and 3' PCR screening. In both Fig 3.1b and c top gel (lane 8), there are no PCR products from the WT DNA template as expected. However, in Fig 3.1b, bottom gel lane 8, the WT had two unexpected size bands from 5' PCR but not from the 3'PCR (Fig 3.1c, bottom gel lane 8). All the PCR reactions shared the same pre-mixture; it was not clear why the WT had unexpected sizes of PCR products.

In Fig 3.1, there is a bright smear band from all the samples running alongside the lowest molecular weight standards. This band is likely to be primer dimer products from primer and is sometimes very strong, as in Fig 3.1b, but sometimes very weak, as in Fig 3.1c. Both the positive control and negative control assure that the PCR screening system is reliable. Subsequently the other putative disruptants were all screened by PCR. The results from this showed that there were 8 mutants from NZE7 transformation: colonies P1-7, P4, P6, P8, 12, P16, P42 and P44; along with 3 mutants from NZE8 transformation: colonies P1-8, P53 and P58. In total, from this preliminary screen, four of these had both 5' and 3' *pksA* integration whilst 7 showed only 5' or 3' integration. Other transformants that were hygromycin resistant but showed no evidence of targeted gene disruption were classified as ectopic transformants.

3.3.2 Purification of the *pksA* mutants

Protoplasts made from mycelium are normally multinucleate. In order to get strains generated from a single nucleus, all the putative *pksA* mutants and five ectopic transformants (P9 and P10 from NZE7 transformation; P19, P83 and P85 from NZE8 transformation) were purified by two rounds of single spore purification.

Fig 3.2 PCR results of the purified strains



General PCR was used to check the purified mutants again with the same primers used to identify the 5' and 3' recombination as shown in Fig 3.1. For each gel, the top gel is to identify the 5' region integration and the bottom gel is for the 3' integration.

The numbers in the parentheses are the sub-strain from the original mutant after single spore purification.

N: negative control (PCR premixture without template DNA)

P1-8: transformants from NZE8

The other mutants shown on the gels are all from NZE7 transformation.

The purified sub-strains of each mutant were tested by PCR again with the same primers using for preliminary screening. A selection of these results is shown in Fig 3.2. After purification, two independent isolates of each of P6 and P1-8 (2 and 3) were shown to be double crossover (Fig 3.2a and b); P8 and P12 (Fig 3.2c) turned out to be 5'single crossover, whilst they were putative double crossover transformants before purification. The other mutants were the same as before. P1-7, P4, P16 and P44 were still 3'single crossover and P42 is 5' single crossover (results not shown). Two other

putative mutants from the second NZE8 transformation were directly checked by Southern blot after single spore purification (P53 and P58).

3.3.3 Southern hybridization to confirm the putative *pksA* mutants

All the single spore purified *pksA* mutants were checked by Southern hybridization. Ectopic transformants and WT (NZE7 and NZE8) genomic DNA were used as controls. The probe was amplified by PCR with primers *R163overlap* and *Dpks1*, which covers the replaced region and 300 bp upstream and downstream of the replaced region. The genomic DNA of each sample was digested with *ScaI* and *KpnI* separately.

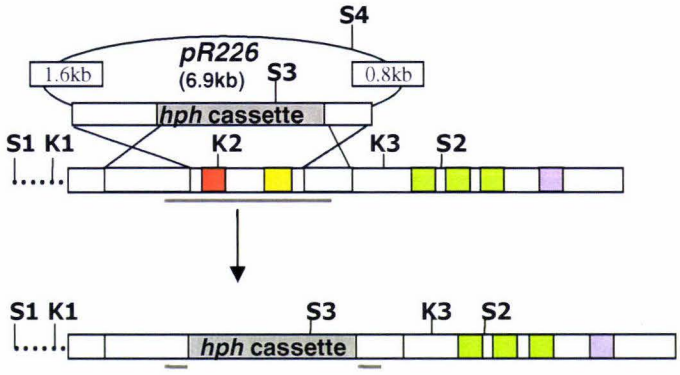
The *ScaI* digestion was used to check the mutants first. There is no *ScaI* in the *pksA* gene targeted region, but two cut sites on pR226: one *ScaI* site is in the *hph* gene region, and another one is on the vector. Therefore, the wild type should give a 14.2 kb band; double cross-over mutants would give two different size bands: a 11.7 kb and a 3.2 kb band. Single cross-over mutants would give three different size bands: a 5'single cross-over would have 11.7 kb, 7.0 kb and 2.5 kb bands and 3' single cross-over would have 13.4 kb, 4.5 kb and 3.2 kb bands from the Southern hybridization (as shown in Fig 3.3a). If a multi-copy integration event occurred, the mutant would have additional bands to those expected.

Some mutants were also checked with *KpnI* digestion. There is no *KpnI* cut site in the *pksA* disruption vector, pR226, but there is a *KpnI* site in the replaced *pksA* gene region. Therefore, Southern hybridization of *KpnI* digestion also can be used to check for double crossover event: one 8.9 kb mutant band instead of two (2.6 kb and 5.5 kb) WT bands. A single crossover event at the 5'or 3'end can be shown by one band size the same as the wild type, and another larger, replacing the second WT band (as shown in Fig 3.3a). If there is a multi-copy integration at 5' or 3' homologous region in the mutants, a large size band (much greater than 12-kb) would be hybridized with the probe.

From the hybridization results shown in Fig 3.3b, WT, P6, P16(1) and P58 had expected size bands of WT, double crossover, 3'single crossover and 5'single crossover respectively. P85, P83 and P47 (2) are ectopic tranformants, they gave ectopic

Fig 3.3 Southern hybridization results of *pksA* mutants

a. Expected hybridisation patterns of *pksA* disruptants patterns of Southern blot



WILD TYPE:

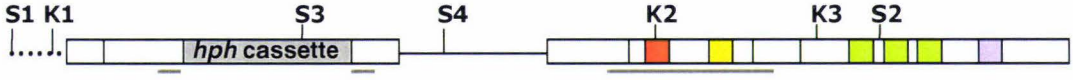
ScaI (S) digestion:	S1-S2: 14.2 kb
KpnI (K) digestion:	K1-K2: 5.5 kb
	K2-K3: 2.6 kb

DOUBLE CROSSOVER:

ScaI (S) digestion:	S1-S3: 11.7 kb
	S3-S2: 3.2 kb
KpnI (K) digestion:	K1-K3: 8.9 kb

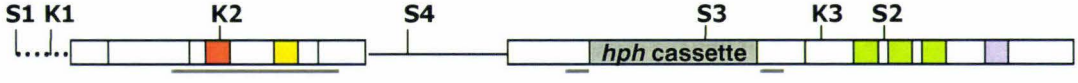
5'SINGLE CROSSOVER: (see diagram below)

ScaI (S) digestion:	S1-S3: 11.7 kb S3-S4: 2.5 kb S4-S2: 7.0 kb	*KpnI* (K) digestion:	K1-K2: 12.6 kb K2-K3: 2.6 kb



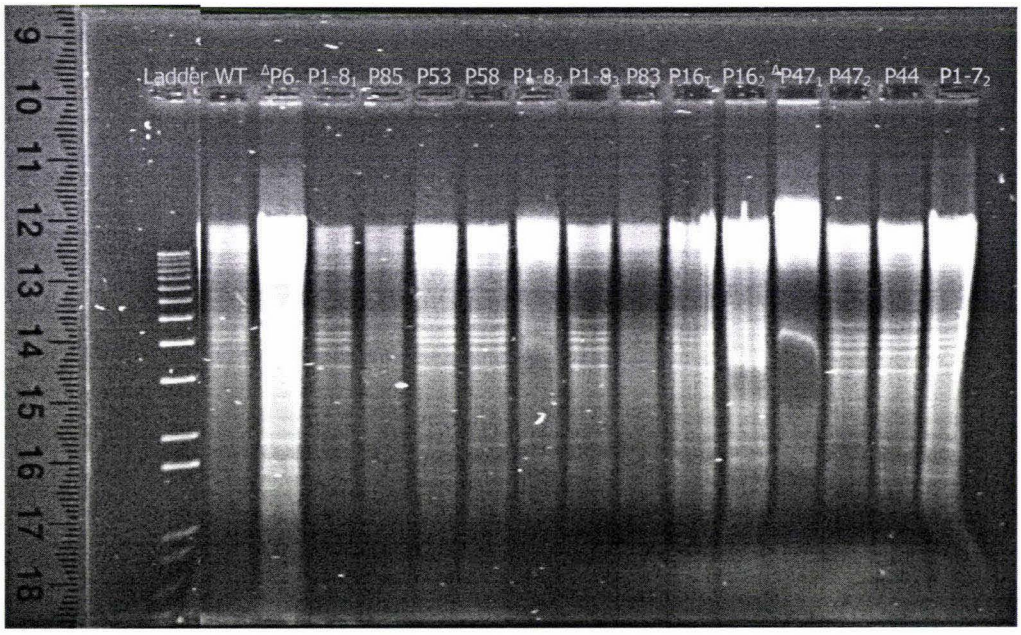
3'SINGLE CROSSOVER: (see diagram below)

ScaI (S) digestion:	S1-S4: 13.4 kb S2-S3: 4.5 kb S2-S5: 3.2	*KpnI* (K) digestion:	K1-K2: 5.5 kb K2-K3: 9.5kb

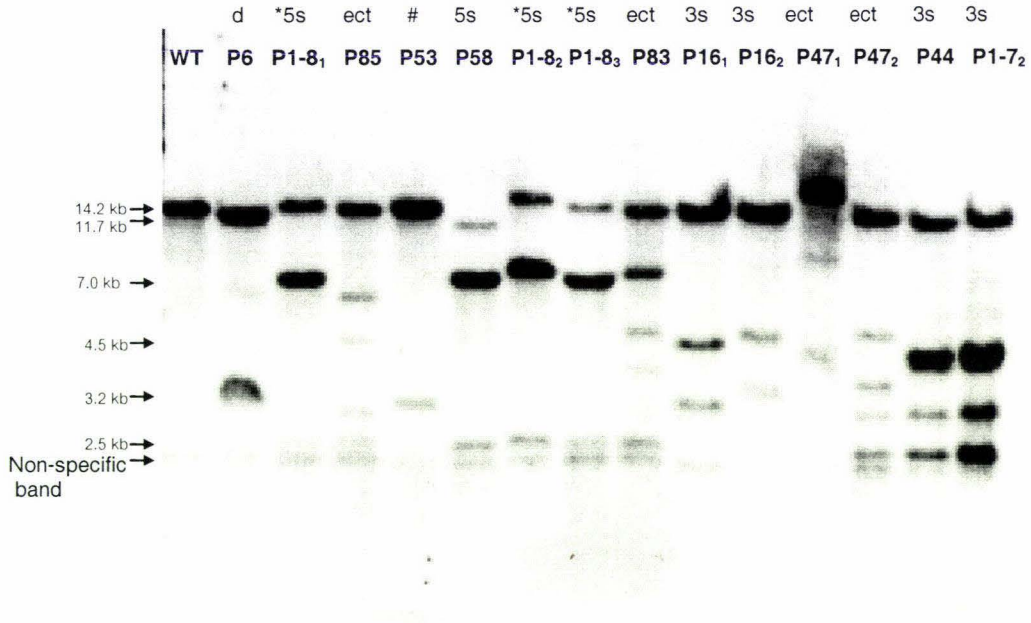


- *pksA* gene region
- 5'or 3'element region
- *hph* gene region
- — *pR226* vector DNA region
- — hybridization probe region
- β Keto acyl Synthase **(KS)** domain
- Acyl Transferase **(AT)** domain
- Acyl Carrier Protein **(ACP)** domain
- Thioesterase **(TE)** domain

b. Southern blot to determine *pksA* mutants with *ScaI* digestion



Δ: $P47_1$ did not digest completely; P6 did not run properly on the gel.

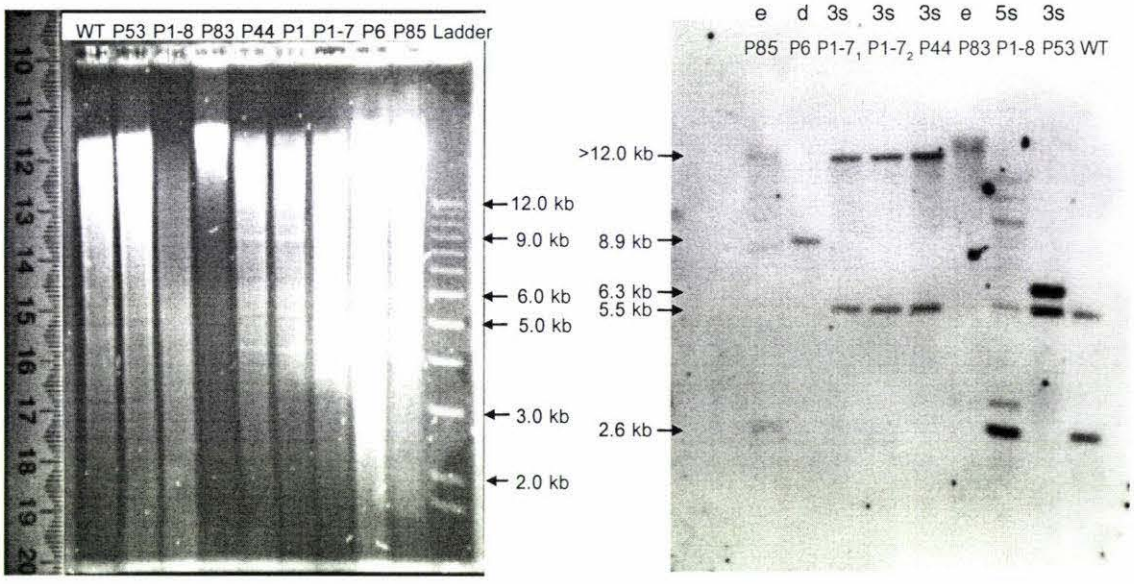


d: double crossover **5s**: 5'single crossover **3s**: 3'single crossover
ect: ectopic transformants **WT**: wild type
* : the results are different from PCR results.
#: unexpected results, it is a mutant but not sure if it is a double crossover mutant.

P85, P53, P58, P83 and P47 are transformants from the second NZE8 tansformation. The sub-strains of each transformant are indicated by subscript numbers.

hybridization bands. P44 and P1-7(2) had more than three bands from the *ScaI* digestion, multi-copy integration event might have occurred in these two mutants. P53 had a ~ 14.2 kb and a 3.2 kb like a double crossover result, but the large band is greater than the P6 large band (11.7 kb). The *ScaI* Southern blot results of the sub-strains from P1-8 (1, 2 and 3) were similar to each other (Fig 3.3b). They gave three different size bands: a ~ 14.2 kb, a 7.0 kb and a 2.5 kb band, which are similar to a 5'single crossover result; but the sub-strains had PCR products from both 5'and 3' region PCR (Fig 3.3b) that indicated they were double crossover. In order to confirm the Southern results of P44, P1-7(2), P53 and P1-8, Southern hybridization with *KpnI* digestion of each samples were carried out (Fig 3.4).

Fig 3.4 Southern hybridization of *KpnI* digestion

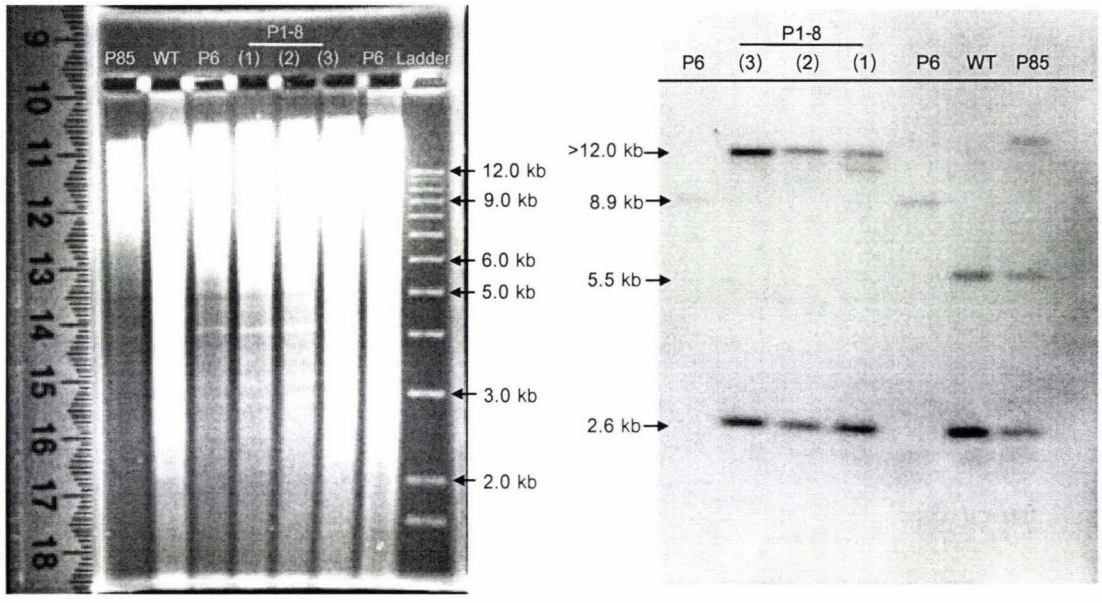


d: double crossover **5s**: 5'single crossover **3s**: 3'single crossover
ect: ectopic transformants **WT**: wild type
P1-7_1 and P1-7_2 are substrains from P-7(NZE7 transformation).
The hybridization band sizes are marked along side the X-ray film picture.

In the *KpnI* Southern blot, both WT (5.5 kb and 2.6 kb bands) and double crossover mutant P6 (8.9 kb) gave expected results. P1-7 and P44 were probably 3'single crossover multi-copy mutants, as seen by the large band much greater than 12-kb. However, P53 had a 5.5 kb band the same as one of the WT bands and a ~ 6.5 kb band smaller than the expected 3' single crossover band size 9.5 kb. This result indicated that P53 is probably also a 3'single crossover mutant; but that somehow the vector region was lost (there were only two bands from the *ScaI* digestion, but the mutant still had

hph resistance). Ectopic strains P85, P83 and mutant P1-8 did not give a good hybridization result. So *KpnI* digestion of P1-8 and P85 was repeated. Before Southern hybridization, in order to check if there was any mistake in PCR, the DNA samples of P1-8 sub-strains were checked by PCR again with the same primers used for checking the purified strains, and the same results as before were obtained (results not shown).

Fig 3.5 *KpnI* digestion to confirm P1-8 mutant



P6: double crossover mutant
P1-8 (1), (2) (3) are substrains from P1-8.
P85: ectopic transformant from NZE8 transformation
WT: wild type NZE8.
The hybridization products sizes are marked alongside the X-film picture.

Southern hybridization results of *KpnI* digestion (Fig 3.5) still support that P1-8 is a 5' single crossover mutant. However, P1-8 (1) has two large bands (>12 kb) and a small band matching the WT. The ectopic strain P85 has two wild type bands and a large ectopic band as expected.

From all the Southern hybridization results, there is one double crossover mutant, P6, five 5' single crossover mutants P1-8, P58, P42, P12 and P8 (Southern blot results of P42, P12 and P8 not shown), and five 3'single crossover mutants, P16, P44, P1-7, P53 and P4. In these ten single crossover mutants, P42, P44 and P1-7 are multi-copy integration mutants, the others are single copy mutants.

3.4 CHARACTERIZATION OF THE *PKSA* MUTANTS

Some of the mutants were studied using TLC and ELISA analysis to check if there was any dothistromin produced by the mutants. Subsequently, an intermediate feeding assay was used to confirm *pksA* function in dothistromin biosynthesis. Whether the mutation had an effect on growth or sporulation was also checked.

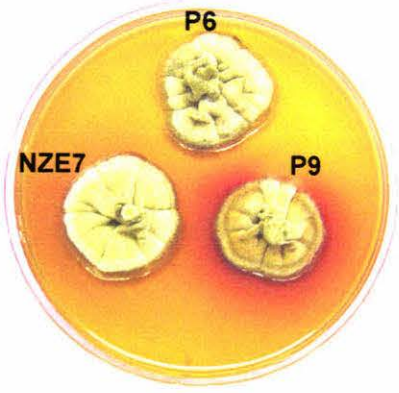
3.4.1 Analysis of dothistromin production of the *pksA* mutants (TLC and ELISA)

In Fig 3.6, the *pksA* mutant P6 is shown on a DM plate. No dothistromin could be seen secreted into the agar, in contrast, dothistromin accumulated (a dark red halo around the colonies) by the ectopic strain P9 and WT NZE7. In the liquid culture (Low DB) colour of the P6 mutant filtrate was shallower than that of WT (Fig 3.6a). Liquid cultures of P6 and NZE7 were extracted (Section 2.12) and run on a reverse phase TLC plate (Fig 3.6b). The TLC results show that there is no red band on P6 lane, corresponding to the strong red band in the wild type and the dothistromin standard (Fig 3.6b); this indicated that there is no dothistromin produced by the knock-out strain. Subsequently, the single cross-over strains were also checked by TLC (Fig 3.6c): three 3' single crossover mutants (P53, P44 and P16) and three 5' single crossover mutants (P42, P12 and P1-8). In Fig 3.6c, the single cross-over strains did not produce dothistromin and any colour compounds. So, there is no any intermediate accumulated in the *pksA* mutants.

Dothistromin production was also analyzed by competitive ELISA as described in Section 2.15 (Fig 3.7), which could quantify the dothistromin concentration. Three replicates of each sample were tested. A serial dothistromin standard dilution was used to prepare a standard curve, which would be used to measure the tested samples dothistromin concentration. The absorbance indicates the dothistromin level in the tested sample, the higher absorbance, the less dothistromin. Negative controls have the greatest value of the absorbance, which was used to calculate the inhibitions of each sample including the standard. The standard curve showed the relationship between the inhibition and the dothistromin concentration. For each standard curve, there is a trend line and the exponential formula of the trend line. The reliability of the trend line was represented by the R (Correlation coefficient) value. The R value closer to 1, the

Fig 3.6 No dothistromin produced by *pksA* mutants

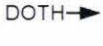
a. Media sample of NZE7 (WT) and P6 (*pksA* mutant)

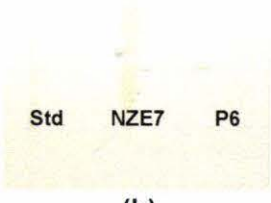


P6 grown on a DM plate, compare to WT NZE7 and ectopic strain P9, there is no dothistromin secreted in the agar.

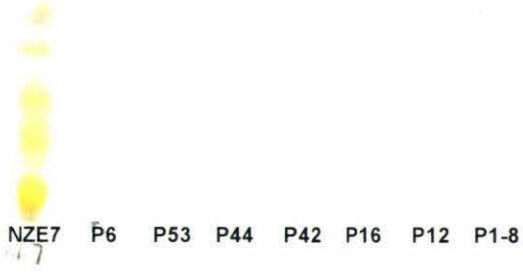
In the liquid culture, the mutant P6 has a shallower colour compare to WT NZE7 there is due to no dothistromin produced in the mutant.

TLC to check intermediates of *pksA* mutants (b) and (c)





(b)



(c)

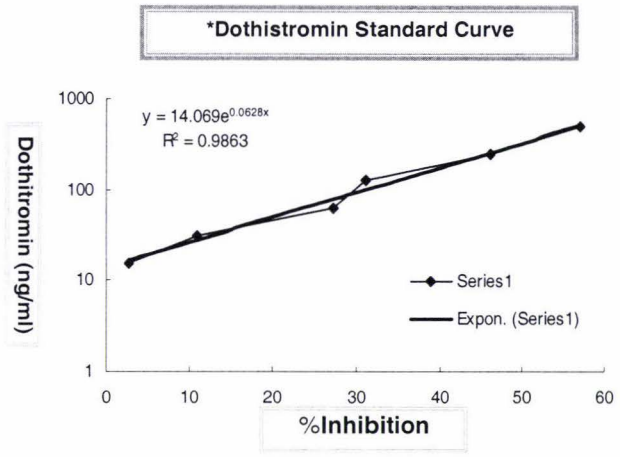
Std: dothistromin standard

DOTH: dothistromin

(b) Intermediates from liquid cultures shown in **(a)**.
(c) Intermediates from the other mutants.

NZE7: wild type
P53: 3'single crossover (without vector region)
P42: 5'single crossover multi-copy
P12: 5' single crossover single copy
P6: double crossover
P44: 3'single crossover multi-copy
P16: 3'single crossover single copy
P1-8: 5'single crossover

Fig 3.7 Competitive ELISA results



Dothistromin standards

Dothistromin (ng/ml)	%Inhibition
15.6	2.8
31.2	11.0
62.5	27.2
125	31.2
250	46.2
500	57.1
1000	59.4

* Dothistromin standard curve data are shown in the right side table.

The results of tested samples

Strain	Absorbance	*% Inhibition	Dothistromin (ng/ml)
Bo (negative control)	**2.293**	**0**	**0**
P6	2.593	-10.7	**0**
P12	2.423	-6	**0**
P16	2.505	-9	**0**
P42	2.527	-10.1	**0**
P44	2.375	-3.6	**0**
P1-8 (1)	2.670	-16.4	**0**
P1-8 (2)	2.605	-13.6	**0**
P53	2.530	-12.9	**0**
WT NZE8 (1:10)	1.561	31.9	**104.3**

%Inhibition was calculated by the formula: **100 × (XB_0 -XB)/XB_0**;
XB_0: the average absorbance of 0 μg dothistromin.
XB is the average absorbance of three replicates of each tested sample.
The higher absorbance, the less dothistromin contained in the sample.

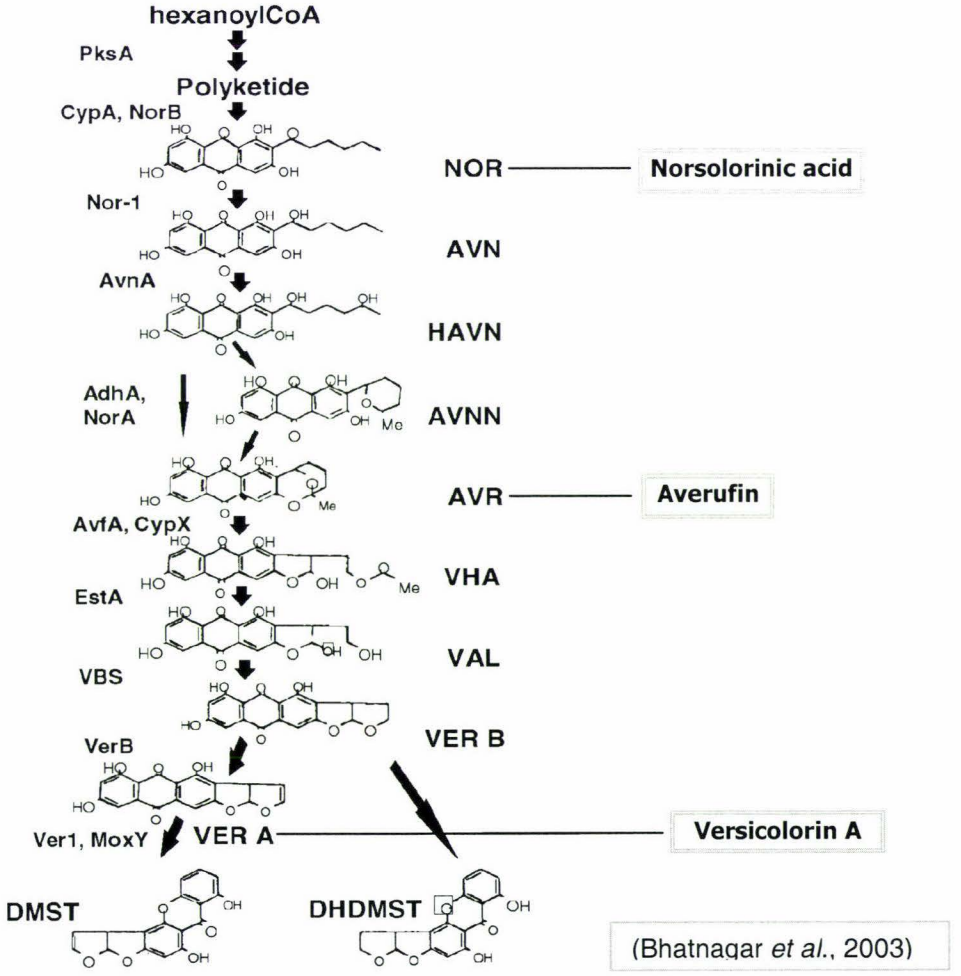
predicted values by the trend line will be closer to the actual values of the tested samples. The dothistromin concentration of each sample was calculated by the inhibition with the exponential formula of the trend line.

All the tested mutants have greater absorbance than the negative control. The inhibitions of the samples are all negative. The ELISA results showed that there was no dothistromin accumulated in the mutants, no matter single crossover or double crossover. These results are consistent with the TLC results. Both TLC and ELISA results showed that there is no dothistromin produced in the *pksA* mutants and indicated that the *pksA* gene is involved in the very early step of the dothistromin biosynthesis.

3.4.2 Intermediate feeding of the *pksA* mutants

To confirm if the *pksA* gene is involved in an early step of dothistromin biosynthesis, four intermediates, NOR, AVR, VERA and AVTH (averythrin) were fed to mutants P6 and P53. As shown in Fig 3.8, NOR, AVR and VERA are precursors of AF. NOR is a very early product of AF biosynthesis, just after polyketide production in *A. parasiticus*. AVR is a substrate of AflI (AvfA) and AflV (CypX); the *aflI* and *aflV* homologs have also been identified in *D. septosporum*. VERA is the first product just after the branch, it is also a precursor of dothistromin (the *dotA* mutant accumulated VERA). AVTH is a metabolite produced by *D. septosporum*, the structure is similar to AVN (averantin, an AF intermediate), and the role in dothistromin biosynthesis is unknown. If the intermediates can rescue the *pksA* gene function in the mutant, the results will confirm that *pksA* gene is involved in the early synthetic step.

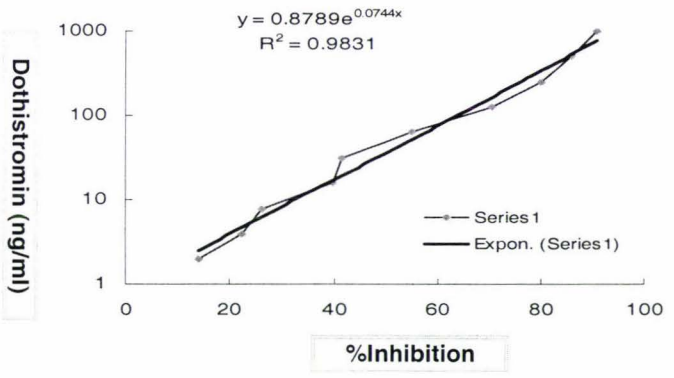
Fig 3.8 AF biosynthesis



The feeding assay was carried out as described in Section 2.14. Two mutants P6 and P53 were used for the feeding study. P6 was fed with all four intermediates. P53 was fed with three intermediates AVR, VERA and AVTH. The cultures were extracted and analyzed by ELISA (Fig 3.9), along with ELISA of the intermediates themselves to test for cross-reactivity. Then the intermediates of P6 feeding were run on a TLC plates (Fig 3.10). The ELISA results showed that NOR and VER A had low cross reactivity with dothistromin antibody, whilst feeding of P6 and P53 indicated that dothistromin production was rescued by feeding. AVR and AVTH had high cross reactivity with dothistromin antibody. So, it is not known if they can rescue the dothistromin production.

Fig 3.9 Intermediate feeding ELISA results

***Dothistromin standard curve**



Dothistromin standard

Dothistromin (ng/ml)	%Inhibition
1.95	14.0
3.9	22.5
7.8	26.3
15.6	39.8
31.2	41.4
62.5	55.0
125	70.4
250	80.1
500	86.0
1000	91.0

* Dothistromin standard curve data are shown in the right side table.

The results of tested samples

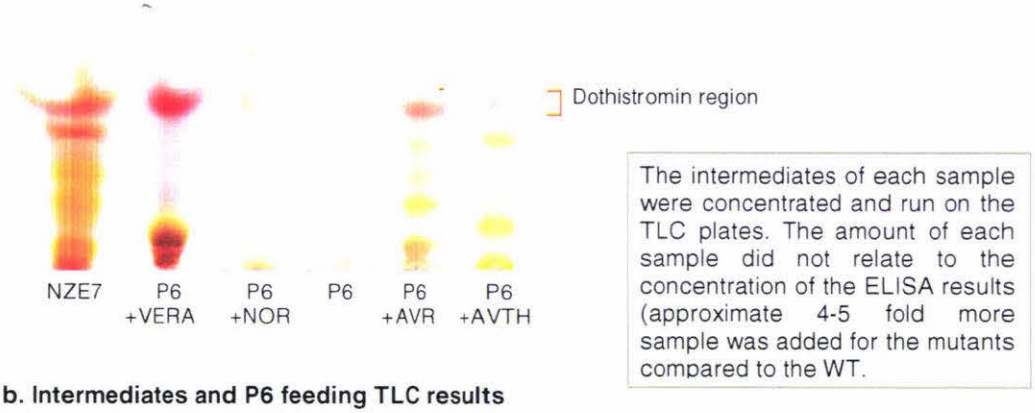
Samples (intermediates & Wild type)	Dothistromin (ng/ml)	Samples (P6 mutant)	Dothistromin (ng/ml)	Samples (P53 mutant)	Dothistromin (ng/ml)
VERA	3.9*	P6+VERA	815.5	P53+VERA	632.8
NOR	5.4*	P6+NOR	214.9	-	-
AVR	105.1*	P6+AVR	223.5	P53+AVR	134.6
AVTH	58.4*	P6+AVTH	333.5	P53+AVTH	110.9
NZE7	4749.9	P6	0.3	P53	1.6

NOR: norsolorinic acid; **VERA**: versicolorin A; **AVR**: averufin; **AVTH**: averythrin.
P6 and P53: mutants; **NZE7**: wild type.
P53 mutant was not fed with NOR due to limited quantities of this intermediate available.
Dothistromin concentration of each samples were calculated by $\mathbf{y = 0.8789e^{0.0744x}}$.
* indicates cross-reactivity of AF intermediates in the dothistromin ELISA.

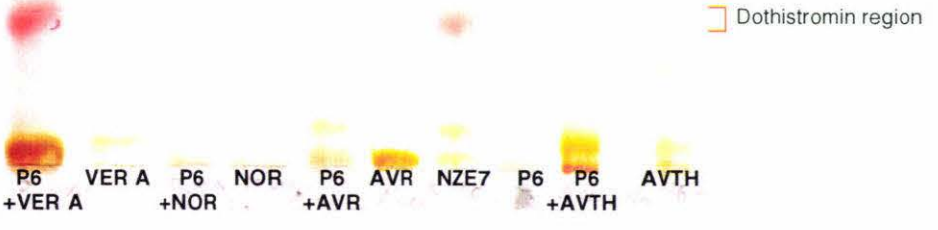
Subsequently, TLC assay was carried out to check if there is any difference between AVR, AVTH and their feeding products. Only the intermediates from P6 feeding assay were checked by TLC, because P6 were fed with four intermediates and have a higher dothistromin production than that of P53. As shown Fig 3.10a, P6 fed with four intermediates all have red band at dothistromin region. Compared to intermediates only on a TLC plate (Fig 3.10b), feedings of P6 with NOR, VERA and AVR showed that dothistromin biosynthesis was rescued. However, AVTH has the same result as feeding of P6 with AVTH, so it is not certain if AVTH can rescue dothistromin biosynthesis.

Fig 3.10 Intermediate feeding TLC results

a. P6 intermediate feeding TLC results



b. Intermediates and P6 feeding TLC results



The feeding of P6 was repeated to confirm that feeding with NOR, AVR and VERA could rescue dothistromin biosynthesis (ELISA). This time, the feeding culture of P6 was directly measured by ELISA before extraction with acetone/chloroform. In order to check the extraction efficiency, the culture after intermediates extraction was also analyzed by ELISA (Fig 3.11) to check if the dothistromin had been extracted out.

3.4.3 Growth rate of the *pksA* mutants

To test whether the *pksA* mutation has an effect on the growth of *D. septosporum*, the radial growth rates of the mutants and WT were measured and compared. On each DM plate, fresh cultures (7 days) of one mutant, one WT and one ectopic transformant colony were inoculated. Colonies were grown on DM plates at 22°C and radial growth measured at 4, 9, 15, 19 and 24 days. Each sample had either 8 or 9 replicates. The data are shown in Table 3.2 (radial growth) and summarized in Table 3.3 (growth rate).

Table 3.2 Radial Growth and unpaired T-test of *pksA* mutants

Strains	4days	9days	15days	19days	24days
	Mean ±SD (mm)	Mean ±SD (mm)	Mean ±SD (mm)	Mean ±SD (mm)	Mean ±SD (mm)
[a] **NZE7** (wild type)	4.5 ± 0.40	8.8 ± 0.82	14.8 ± 1.03	19.2 ± 0.96	23.6 ± 1.19
[a] **P6** (double cross-over)	4.3 ± 0.41	8.0 ± 0.82	14.2 ± 0.83	*18.1 ± 0.77	23.0 ± 1.28
[a] **P9** (ectopic)	4.3 ± 0.38	*7.9 ± 0.70	14.0 ± 0.68	*17.4 ± 0.65	*21.2 ± 0.81
[b] **NZE8** (wild type)	4.4 ± 0.46	8.9 ± 0.46	14.6 ± 1.18	18.6 ± 1.02	23.1 ± 0.98
[b] **P53** (3'single cross-over)	4.0 ± 0.36	*8.3 ± 0.66	14.0 ± 0.57	17.8 ± 0.66	22.3 ± 0.69
[b] **P19** (ectopic)	4.1 ± 0.41	*7.9 ± 0.72	14.0 ± 0.78	17.9 ± 0.67	22.3 ± 0.95

a: n=9; P6, P9, NZE7. P6 and P9 are transformants from NZE7 transformation; their growth was compared to WT NZE7's growth.

b: n=8; P53, P19, NZE8. P53 and P19 are transformants from NZE8 transformation; their growth was compared to WT NZE8's growth.

*: indicates a statistically significant difference from the wildtype.

Table 3.3 Growth Rate of *pksA* mutants

Strains	Growth Rate (mm/day)	Unpaired *T-test* of *pksA* mutant/WT	
	Mean ± SD	*T*	*p*
[a] **NZE7** (wild type)	0.96 ± 0.06	-	-
[a] **P6** (double cross-over)	0.94 ± 0.05	0.795	0.438
[a] **P9** (ectopic)	0.84 ± 0.05	4.52	0.0003*
[b] **NZE8** (wild type)	0.93 ± 0.04	-	-
[b] **P53** (3'single cross-over)	0.91 ± 0.03	1.096	0.292
[b] **P19** (ectopic)	0.91 ± 0.05	1.001	0.334

a: n=9; P6, P9, NZE7. P6 and P9 were compared to NZE7.
b: n=8; P53, P19, NZE8. P53 and P19 were compared to NZE8.
*: indicates the data statistically significant.

In Table 3.2 (Radial Growth), for the NZE7 transformants, the ectopic strain P9 had statistically significantly smaller radial growth when compared to WT on days 9, 19 and 24. The mutant strain P6 also showed slower growth on day 19. For NZE8 transformants, the mutant strain P53 and the ectopic strain P19 were both slower than WT at day 9 but not at the other time points. Therefore the overall growth rate for each strain was carried out by calculating average of the growth per day from 5 to 24 days (Table 3.3). In Table 3.3, only the ectopic strain P9 showed significantly slower growth ($p = 0.0003$), the other strains showed similar growth rate to WT. Therefore, from Table 3.2 and Table 3.3, the data indicated that the *pksA* mutations had no overall effect on the colony growth rate.

3.4.4 Sporulation of the *pksA* mutants

In *A. nidulans,* conidiation is associated with production of the sterigmatocystin, (Wilkinson *et al.*, 2004). Therefore, whether the *pksA* gene mutants had reduced sporulation was checked by growing the mutant strains on sporulation plates (DSM plates) and the spore concentration of a fixed area of mycelium was determined using a haemocytometer. Six *pksA* mutants were tested: One double cross-over P6, three 5'single cross-over, P8, P58 and P1-8; and two 3'single cross–over P16 and P53; along with two ectopic strains P10 and P19 and two wild type isolates NZE7 and NZE8. Five replicates of NZE7 transformants: P6, P8, P16 and P10 were checked and four replicates of NZE8 transformants: P53, P58, P1-8 and P19 were checked. The results were analyzed using an unpaired t-test (http://www.graphpad.com) comparing each strain to the wild type and are shown in Table 3.4.

All the *pksA* mutant strains: P6, P8, P16, P53, P58 and P1-8 showed significantly reduced ($p< 0.05$) sporulation (by approximately 60-70%) compared to WT strains: NZE7 and NZE8. In contrast, the ectopic strain P10 had increased sporulation, although it is not statistically significant ($p = 0.05$). The sporulation rate results suggested that the *pksA* mutants reduce the sporulation significantly and support the view of a relationship between secondary metabolites and sporulation.

Table 3.4 Sporulation of *pksA* mutants

Strain	Spores×10^5 per ml	Unpaired *T-test* of *pksA* mutant/WT	
	Mean ± SD	*t*	*p*
[a] **P6** (double cross-over)	10.17 ± 5.22	4.76	*0.0014
[a] **P8** (5'single cross-over)	8.22 ± 2.35	6.26	*0.0002
[a] **P16** (3'single cross-over)	7.27 ± 1.65	6.74	*0.0001
[a] **P10** (ectopic)	41.5 ± 10.55	2.29	0.0506
[a] **NZE7** (wild type)	28.56 ± 6.87	-	-
[b] **P53** (3'single cross-over)	9.36 ± 4.09	4.06	*0.0067
[b] **P58** (5'single cross-over)	12.18 ± 2.36	3.72	*0.0098
[b] **P1-8**(5'single cross-over)	7.49 ± 1.97	4.65	*0.0035
[b] **NZE8** (wild type)	31.75 ± 10.24	-	-

a: n=5; P6, P8, P16, P10, NZE7. P6, P8, P16 and P10 were compared to NZE7 sporulation.

b: n=4; P53, P58, P1-8, NZE8. P53, P58, P1-8 were compared to NZE8 sporulation. There were no spores from the ectopic strain P19, which was not included in the table.

*: indicates the data statistically significant.

3.5 DISCUSSION

Based on the structural similarity of dothistromin to the polyketides aflatoxin and steigmatocystin, and the identification of *pksA* gene, dothistromin biosynthesis has been proposed to be a polyketide pathway. However, experimental evidence of this hypothesis had not been obtained. In this study, the role of *pksA* in dothistromin biosynthesis was determined by gene disruption. The results demonstrated that the *pksA* gene product is required for dothistromin biosynthesis and it functions similar to its homologs in AF/ST biosynthesis.

3.5.1 Transformation

The *pksA* mutants were obtained by PEG/protoplasts transformation which was also used for *dotA* gene disruption (Seconi, 2001). However, it was not initially successful when used in attempts to transform the *pksA* disruption vector pR226 (Teddy, 2004). The transformation with pR226 was continued in this project and was repeated several times before a successful transformation. In this work several alterations were made to the transformation methodology used by Teddy (Teddy, 2004): the incubation time for

protoplasts isolation was increased from 3 to 12-16 hours and a different source of Glucanex cell wall-lysing enzyme was used.

Five-μg vector DNA was used for the transformation. The transformation results suggested that the DNA concentration affected the transformation frequency. The higher the DNA concentration used in transformation, the more transformants were obtained. This result may be due to the different volumes of the DNA vector used in the transformation. The lower concentration of the vector DNA, the higher volume is required for the transformation. After mixing with the protoplasts, too much DNA solution diluted the transformation mixture system and caused damage to protoplasts and lead to less transformants.

It was reported that there is no difference in the transformation frequency when circular or linear DNA is used for transformation (Fincham, 1989). Circular vector DNA had been successfully used to obtain *dotA* replacements (Seconi, 2001). In this study, circular DNA was also directly used in the transformation. There were 50 transformants of the *pksA* transformation (NZE7 transformation, Table 3.1 Second row). Of these, there were 8 *pksA* targeted disruptants obtained and the targeting frequency was 16% (*pks* mutants verse total transformants). However, there was only one double crossover gene replacement (homologous recombination at both homologous regions) out of the 8 *pksA* mutants. The transformation frequency was reasonable. Nevertheless, the only one replacement of the *pksA* was unexpected.

It was thought that the recombination efficiency was related to the length of the homologous region of the circular vector DNA (Bird and Bradshaw, 1997). The homologous regions used for gene disruption are normally over 2 kb in filamentous fungi. For example, 2.6 kb and 1.6 kb homologous regions were used in the *CTB1* gene disruption, to obtain 16 mutants out of 41 transformants (Choquer *et al.*, 2005). In *stcL* gene disruption, a 4.8 kb homologous region was used and yielded 8 replacement out of 36 transformants (Keller and Hohn, 1997). Compared to them, the homologous regions (1.3 kb and 0.83 kb) used in the *pksA* gene disruption were relatively short; this may have contributed to the low double cross over recombination efficiency.

It was also reported that for homologous recombination, linear DNA had greater target

disruption efficiency than circular DNA (Bird and Bradshaw, 1997). Another report considered that cutting the disruption vector in the homologous-coding region can lead to high integration at the homologous locus (Fincham, 1989). Therefore, the low double crossover event may have been partly due to transformation without cutting the vector in the homologous *pksA* coding region. Both the use of circular vector and the short homologous regions in the disruption vector pR226 may therefore account for the high proportion of single crossover (5'or 3') gene disruptions obtained compared to a single double crossover gene replacement.

3.5.2 Identification of the mutants

The putative *pksA* mutant can be distinguished by the lack of dothistromin production. The homologous recombination of putative mutants was pre-screened by PCR with the mutants' genomic DNA, and it worked well. Since the protoplasts were made from mycelia, the transformants of the protoplasts might be multinucleate. Two-round single spore purification of the mutants could obtain a pure homokaryotic strain. The purified mutants may have different results from the pre-screening. For example the mutant P8 appeared to be a double crossover mutant (Fig 3.1b and c) in the preliminary screening PCR, but a 5'single crossover mutant (Fig 3.2c) after purification.

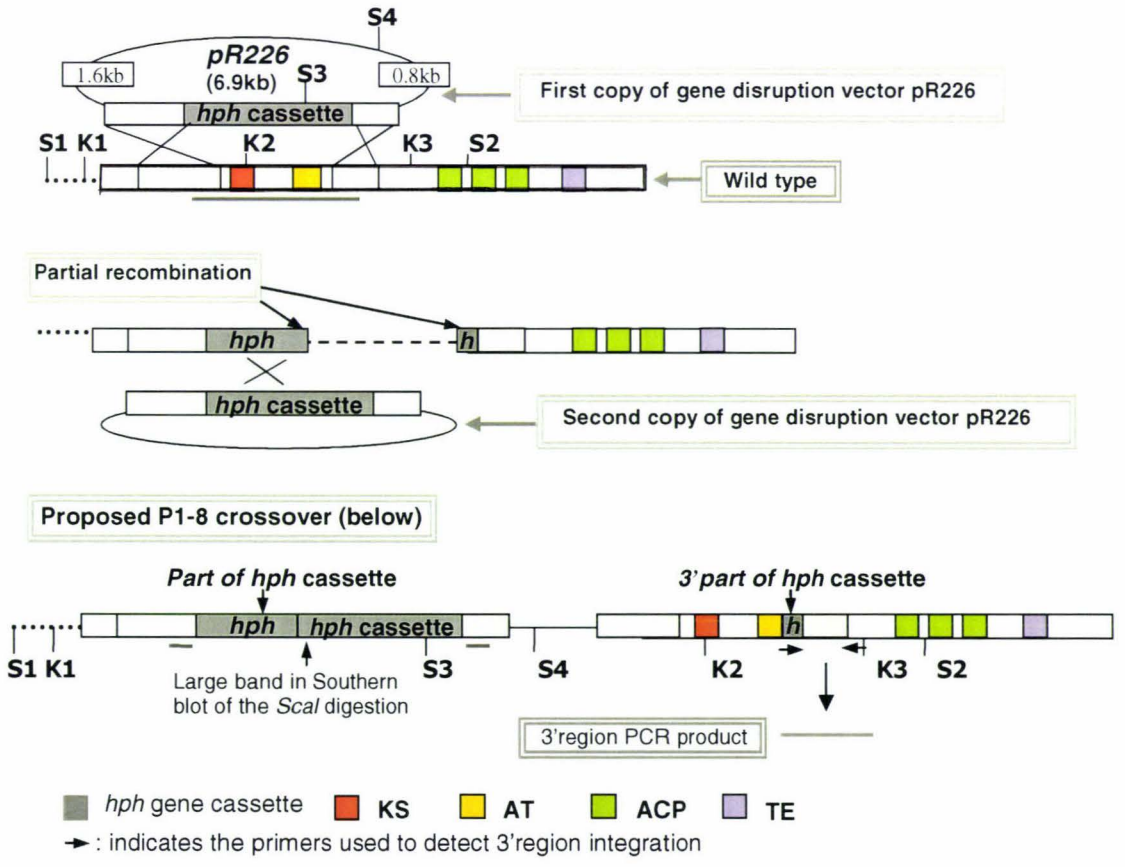
The mutants were analyzed by Southern hybridization to check the integration on the genome. The probe was designed to cover 300 bp of both homologous regions in the disruption construct. The probe would be able to identify any integration of the disruption vector in the *D. septosporum* genome. The *ScaI* and *Kpn I* digestions were used to identify the difference between the mutants and the WT.

From the Southern hybridization results, most of the mutants gave expected results. But the mutant P53 had unexpected hybridization bands in both of the *KpnI* and *ScaI* digestions. In the *ScaI* digestion hybridization results, P53 was thought to be a double crossover mutant, since it had two bands and a single crossover mutant should give three bands. However, the *KpnI* digestion hybridisation results showed that P53 was a 3'single crossover mutant, with a 5.6 kb WT band, but another band smaller than the expected size. From this result, two different enzyme digestions for checking the mutant are necessary.

Another unexpected hybridization result was obtained from the mutant P1-8. It appeared to be a double crossover in the preliminary screening PCR and the PCR after single spore purification. However, the Southern blot results of both *ScaI* and *KpnI* digestions showed that P1-8 was a single crossover mutant. It is the only mutant that the Southern hybridization gives different results from the PCR with the purified strain's DNA. After hybridization of the *ScaI* digestion, the P1-8 genomic DNA was re-checked by PCR and still shown to be a double crossover mutant, but the following hybridization with the *KpnI* digestion still suggested that P1-8 is a single crossover.

However, in the hybridization results of both *ScaI* and *KpnI* digestion of mutant P1-8, the band sizes are larger than expected. In the *ScaI* digestion, it had a similar size band as WT (~14 kb) instead of a 11.7 kb 5'single crossover band; in the *KpnI* digestion, the 5'end band size was also greater than 12.6 kb. There could have been an extra ~2.0 kb fragment integration occurring in the 5' region, that fragment does not have the *ScaI* cutting site. Both the 5'and 3' region PCR gave expected size products indicating that *hph* gene fragments integrated in the genome as double crossover as shown in Fig 3.12.

Fig 3.12 Possible integration of P1-8



It was reported that homologous integration between a disruption vector and the recipient chromosome was through the DNA repair system (Fincham, 1989). There is a possibility that homologous recombination occurred at both 5' and 3' elements region in P1-8, but somehow, the recombination was not completed. Under hygromycin B resistance selection, a second copy of the disruption vector may have recombined with the first copy at the *hph* homologous region to rescue the *hph* gene cassette. Therefore, the mutant P1-8 still gave both 5' and 3' region PCR products but showed single crossover mutant in Southern hybridization with a larger size band than expected.

In the screening method used in this study, PCR is very efficient and suitable for the preliminary screening, but for determination of the mutants, Southern hybridization is required to confirm the recombination event in the genome.

3.5.3 Characterization of the *pksA* mutants

The appearance of confirmed mutants had no differences from the WT, except for loss of dothistromin pigmentation. The mutants were characterized by reverse phase TLC and competitive ELISA assays. Both assays have been successfully used to analyze the *dotA* mutants (Bradshaw *et al.*, 2002). They have different functions in mutant characterization. TLC assay could be used to detect intermediates accumulated in the mutants and the ELISA assay could be used to quantify the dothistromin produced by the mutants. Both of them showed that there is no dothistromin produced by any of the mutants.

All the *pksA* mutants gave greater absorbance values than the negative control in ELISA (Fig 3.7) leading to negative %inhibition values. The same phenomenon was observed in an ammonium media ELISA in a previous study and it was thought to be caused by enhancement of the alkaline phosphatase (linked to the antibody) by ammonium (Ganley, 2000). In this study, although there is no ammonium added in the medium, the negative values of the mutants may be caused by the interaction of metabolites accumulated in the mutants.

The results of TLC and ELISA showed clearly that the *pksA* gene is required for dothistromin biosynthesis. The intermediates samples of the mutants on the TLC plates

(Fig 3.6c) provide evidence for the role of *pksA* early in dothistromin biosynthesis prior to any coloured compound formation. The function of *pksA* is similar to its homologs, *aflC* (*pksL1*) (Feng and Leonard, 1995) and *stcA* (Yu and Leonard, 1995), in the AF/ST cluster.

Feeding with the putative downstream intermediate NOR (an early product of AF biosynthesis) confirmed that the *pksA* gene product is involved in a very early step of dothistromin biosynthesis prior to formation of NOR. This result implied that NOR, or a very similar compound must be an early intermediate of dothistromin biosynthesis. In the feeding assay, VERA could also rescue dothistromin biosynthesis. This was expected, since it is an intermediate accumulated by *dotA* mutants. AVR and AVTH were extracted from *D. septosporum* cultures, but it is not known if they are precursors of dothistromin. Feeding of the P6 mutant with AVR produced dothistromin, suggesting that AVR is a precursor of dothistromin. The intermediate AVTH has a similar structure to AVN, a product that follows NOR in AF biosynthesis. However, AVTH interacted strongly with the antibody used to detect dothistromin by ELISA and had similar result as feeding of P6 with it on a TLC plate (Fig 3.10b), so no conclusion could be drawn with this metabolite.

3.5.4 The relationship of secondary metabolites and sporulation

Radial growth rates of the mutants were characterized. There was no difference between the *pksA* mutants and the wild type. However one of the ectopic strains, P9, had significantly slower growth compared to WT. This mutant also showed higher dothistromin production compared to wild type on DM plates (Fig 3.6a). The integration of DNA in this ectopic transformant may have occurred at a region which regulated the dothistromin production and the growth of the strain.

It was previously reported that secondary metabolite production is sometimes associated with sporulation (Wilkinson *et al.*, 2004). The inability of the *pksA* mutants to synthesis dothistromin provided a chance to analyse the relationship between dothistromin production and the sporulation rate. All the mutants showed lack of dothistromin production and six of them were chosen for the sporulation assay. The mutants showed a significant (60-70%) reduction of sporulation compared to WT, whilst the ectopic

strain P10 with strong dothistromin production had slightly more spores than that of WT NZE7. The results indicated that there is a relationship between dothistromin production and the sporulation rate in *D. septosporum*, and this result is similar to the relationship between the sporulation and ST production in *A. nidulans.*

Some fungal secondary metabolites have been shown to be associated with virulence. The gene disruption of *CTB1* in *Cercospora nicotianae* led to mutants deficient in cercosporin production that exhibited lower virulence on tobacco leaves (Choquer *et al.*, 2005). Unfortunately, due to lack of an efficient pathogenic test of *D. septosprum* to its host, the relationship between virulence and dothistromin production could not be tested. Another student in our lab is trying to develop this system. However, the *pksA* mutants will provide a negative control to test if dothistromin is required in the infection of the host, *P. radiata.*

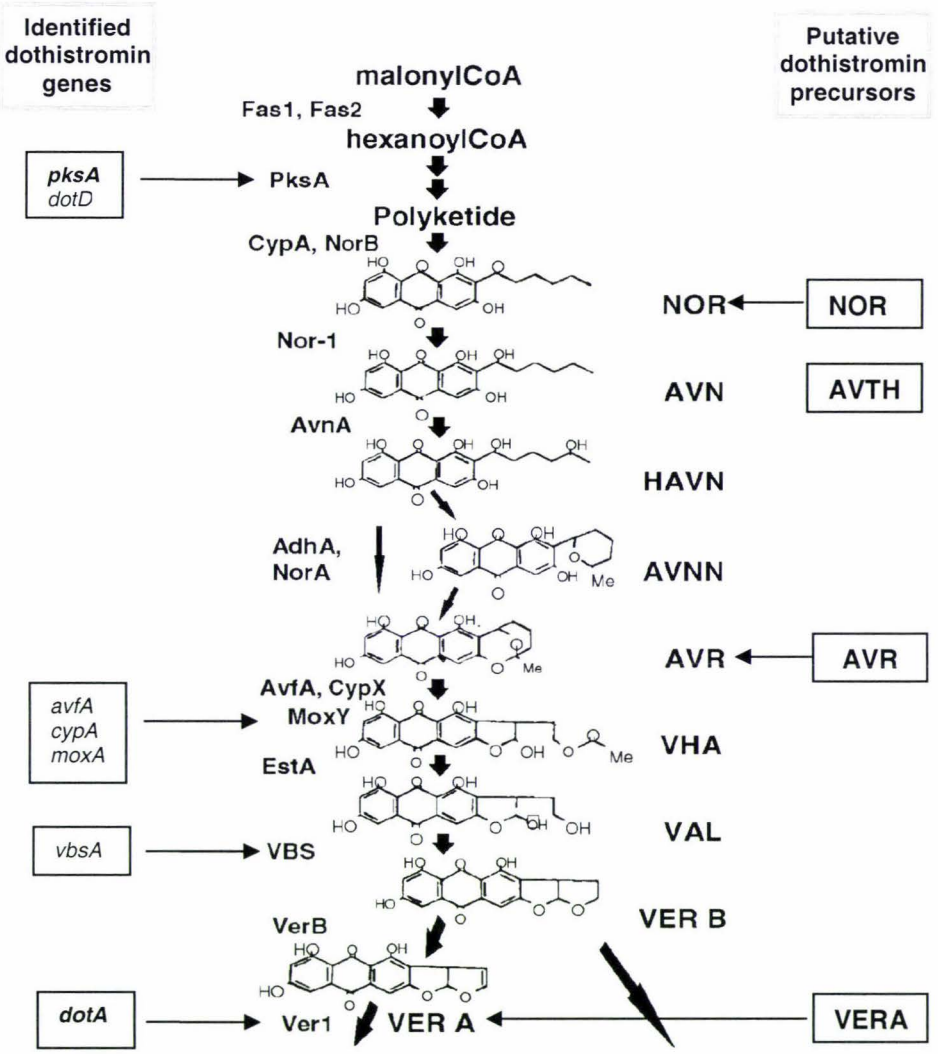
3.5.5 Dothistromin gene cluster

The *pksA* gene disruption and the intermediate feeding of the *pksA* mutants showed that PksA function was extremely similar to its homologs in AF/ST biosynthesis. The *dotA* mutant also demonstrated similar function to its homologs in the AF/ST cluster (Bradshaw *et al.*, 2002). The functions of the two dothistromin biosynthesis genes implied that the AF/ST gene cluster will be very helpful to identify further dothistromin biosynthesis genes. At least, it is very likely that dothistromin shares biosynthetic steps with AF before the branch point (*aflW, ver-1*) of AF biosynthesis (Fig 3.13). The intermediates used in the feeding assay also provided a clue that NOR and AVR may also be produced during dothistromin biosynthesis. The genes encoding enzymes for the synthesis and modification of these intermediates should be present in the dothistromin cluster. This hypothesis is supported by the identification of a putative *vbs* (versicolorin B synthase) gene in *D. septosporum* in our lab.

Given that dothistromin is a polyketide, the Pks*A* functions as the AflC/StcA in AF/ST biosynthesis using short chain fatty acid as a start unit to form polyketide backbones (Feng and Leonard, 1995; Yu and Leonard, 1995). There is a possibility that the short chain fatty acid is synthesized by fatty acid synthase (*fas*) genes, which are also required in dothistromin biosynthesis. The *fas* genes are also highly conserved in fungi. It may

be possible to use conserved domains of FAS to identify fatty acid genes in dothistromin biosynthesis. According to AF biosynthesis, there may be at least 8 genes in the partial pathway of dothistromin biosynthesis as shown in Fig 3.13.

Fig 3.13 The putative dothistromin biosynthetic steps



modified from (Bhatnagar *et al.*, 2003)

The putative dothistromin genes and their corresponding homologous gene products in AF biosynthesis are indicated by arrows and lists at left hand; the intermediates of the dothistromin biosynthesis and the formation step in AF biosynthesis are point out. AVTH has the similar structure to AVN in AF biosynthesis, it is showed beside AVN.

The genes, *pksA* and *dotA* with bold letters indicate that they were characterized by gene disruption and they are involved in dothistromin biosynthesis.

NOR, norsolorinic acid; AVN, averufanin; AVR, averufin; AVTH, averythrin; VER A, versicolorin A.

CHAPTER FOUR: *EPOA* GENE DISRUPTION

4.1 TARGETED REPLACEMENT AND CHARACTERIZATION OF A PUTATIVE DOTHISTROMIN BIOSYNTHETIC GENE, *EPOA*

As mentioned in the introduction, the putative *epoA* gene is the only one that has no homolog in the AF/ST gene cluster amongst the identified dothistromin genes so far. Whether it is involved in dothistromin biosynthesis is unpredictable. It may be involved in a late step of dothistromin biosynthesis or it may not be a dothistromin gene. Targeted gene replacement of *epoA* will reveal the gene function.

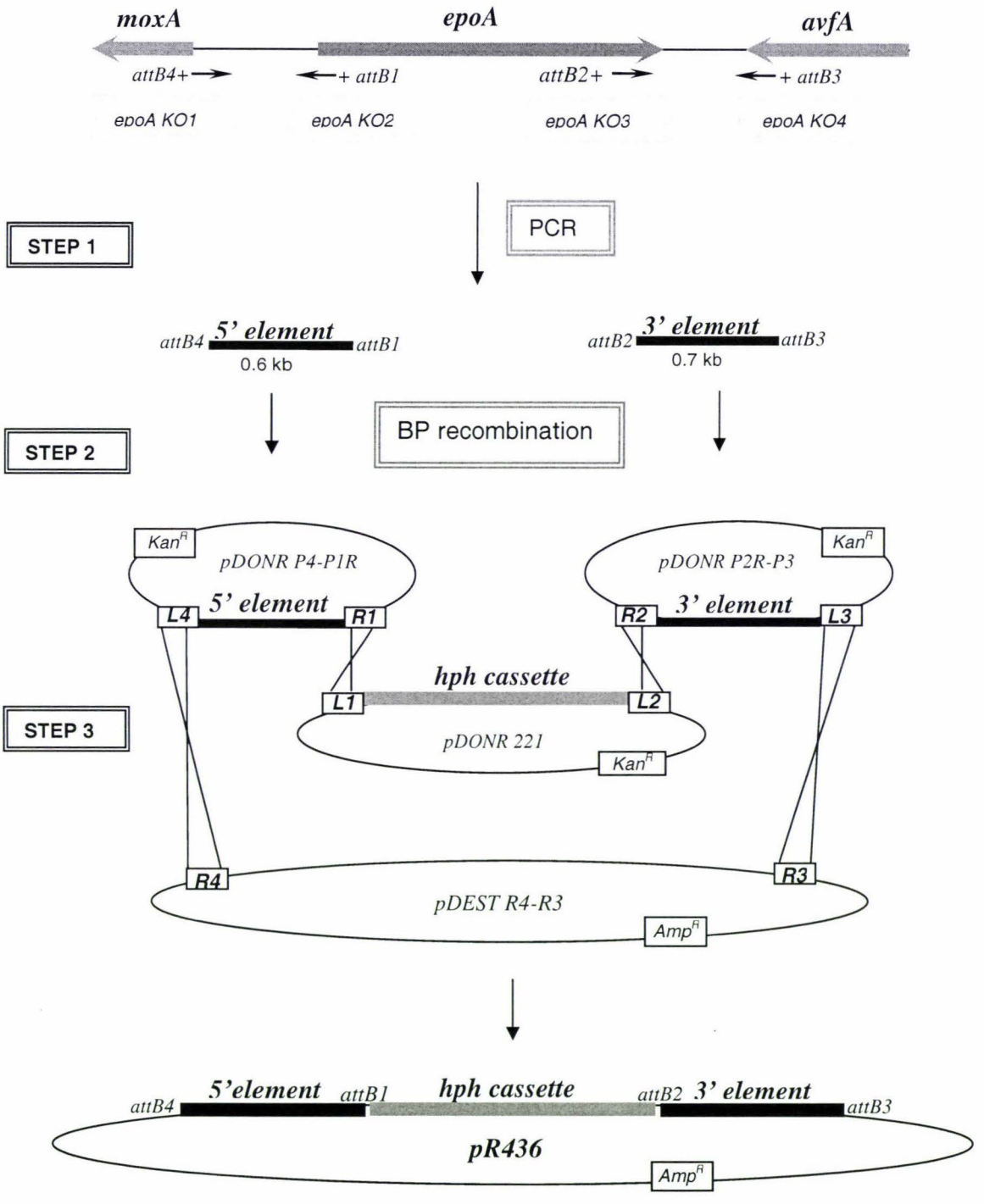
For the targeted gene replacement, it is necessary to construct a disruption vector, transform *D. septosporum*, confirm the targeted disruption and characterize the mutant strains.

4.2 CONSTRUCT *EPOA* GENE DISRUPTION VECTOR

The *epoA* gene disruption vector was constructed using the Gateway system (Section 2.4.3), which provides a method to construct multiple DNA fragments in one vector. As mentioned in the introduction (Section 1.3.1), the Gateway system was carried out with two recombination reactions of *att* sites. Firstly, regions flanking *epoA* were PCR amplified using primers with *attB* recombination sites attached. Secondly, BP recombination allowed PCR products flanked with *attB* sites to recombine with suitable donor vectors to generate three entry clones: a 5'entry clone, a 3'entry clone and a selectable marker gene entry clone. Thirdly, LR recombination allowed these three entry clones to recombine with a destination vector to create the final gene disruption vector.

In each recombination, the entry fragments replaced the *ccdB* gene, a negative selection gene, and a gene that confers chloramphenicol resistance. The CcdB protein inhibits the growth of most *E. coli* strains by interfering with *E. coli* DNA gyrase, so, only the recombinant vector can be generated after transformation in certain *E. coli* strains. Fig 4.1 shows the positions of primers used for the construction of an *epoA* disruption vector using the Gateway system.

Fig 4.1 Experimental procedure for constructing the disruption vector



The primer arrows indicate the approximate location of the primers used for amplifying the 5'and 3'element.

4.2.1 Step 1 PCR to add *attB* sites

As shown in Fig 4.1, the putative dothistromin genes, *moxA, epoA* and *avfA* are very close to each other on the genome. There is only 530-bp between the start codons of *epoA* and *moxA* and 177-bp between *epoA* and *avfA* stop codons. Primer *epoAKO1* (for amplifying the 5' flanking element) was designed just upstream before the *moxA* coding region and primer *epoAKO4* (for amplifying the 3'flanking element) was designed in 3' intron region of the *avfA* gene. Primers *epoAKO2* and *epoAKO3* were designed in the *epoA* gene to replace ~ 800 bp coding region (most of the conserved domain EHN) of the *epoA* gene in future. The sequences of primers *epoAKO1, epoAKO2, epoAKO3* and *epoAKO4* are shown in Table 2.2, the precise location of these primers is shown in the Appendix III.

The 5' and 3'element regions that flank the *epoA* gene were amplified by PCR with primers tailed with the correct *attB* sites. As shown in Fig 4.1, the primers *epoAKO1* and *epoAKO2* were used to amplify genomic DNA to give a 0.6 kb fragment of the 5'element and the primers *epoAKO3*and *epoAKO4* were used to amplify genomic DNA to give a 0.7 kb fragment of the 3'element. The *attB4* and the *attB1* sites for generating a 5' entry clone by recombination were added at the 5'ends of primers *epoAKO1* and *epoAKO2*. The *attB2* site and the *attB3* site for generating a 3' entry clone were added at 5'end of primers *epoAKO3* and *epoAKO4*.

The selectable marker gene entry clone, pR225, was generated by amplifying the hygromycin (*hph*) gene with *attB1* and *attB2* tailed primers followed by recombination into donor vector pDONR221; this construct was prepared by Teddy (Teddy, 2004). The *hph* gene was used as the dominant selectable marker in *D. septosporum* transformation.

4.2.2 Step 2 BP (attB: attP) recombination to generate entry clones

The PCR products with correct *attB* sites attached to the 5'element and 3'element were transferred into pDONR^{TM} vectors by BP recombination reactions (Section 2.4.3.1). Each of the pDONR^{TM} vectors contains an *attP* region, which allows recombination with the appropriate *attB* sites of the PCR products. The 5' and 3'elements and their

corresponding donor vectors are shown in Table 4.1. After BP recombination, each entry clone contains *attL* and *attR* recombination sites for the next step (LR recombination).

Table 4.1 The 5' and 3' elements constructs

PCR product	Donor vector	Entry clone
attB4-5'element-attB1	p DONR P4-P1R (Kana^R)	p5'element
attB2-3'element-attB3	p DONR P2R-P3 (Kana^R)	p3'element

The 5' element and 3'element BP reaction systems were treated with proteinase K and transformed into TOP 10 *E. coli* $CaCl_2$ competent cells (Section 2.5.1.2). The 5'element produced 4 transformants, but the 3'element produced none. Subsequently the 3'element BP reaction was cleaned using a PCR purification column, and transformed by electroporation into TOP 10 electroporation competent cells. This procedure resulted in twelve 3'element transformants. Four colonies of each type of transformant were checked by PCR. The 5'element colonies were checked with primers *M13reverse* and *R162areverse5*, and the 3' element colonies were checked with primers *M13reverse* and *R162areverse2*. The R162a reverse primers were in the 5'and 3' element regions and the M13 primers annealed to the Donor vector sequence. Three of 4 colonies of the 5'element BP reaction and all of the 3'element colonies gave PCR products of the expected size (results not shown). One of each positive clone of the 5'element and 3'element was used for the preparation plasmid DNA, sequenced and used for LR recombination.

4.2.3 Step 3 LR recombination to create final disruption vector pR246

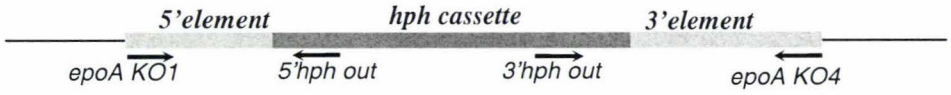
As shown in Fig 4.1 step 3, LR recombination occurred at the homologous *attB* sites: L4-R4, R1-L1, R2-L2, and L3-R3. The three entry vectors (60 ng of each vector) were added to 60 ng pDEST R4-R3 vector. After recombination, the mixture was cleaned on PCR purification column (QIAGEN) and 5µl of the purified DNA was used for *E. coli* transformation. Hundreds of colonies were generated on the LB plates (*Amp^R*).

Negative selections with the *ccdB* gene and ampicillin resistance were used to select *E. coli* with the destination vector containing the three-way recombination. Theoretically,

only transformants with the correct recombination should be obtained. Five randomly picked transformants were checked by PCR with two primer pairs: *epoAKO1* and *5'hphout;* and *3'hphout* and *epoAKO4*. These primers identified correct recombination of the *hph* cassette with the 5'element and 3'element respectively, as shown in Fig 4.2a. Only when three-way LR recombination occurred, would PCR give products with both of these primer pairs.

Fig 4.2 LR recombination

a. The diagram of the LR recombination and the primers location



Approximate positions of primers used for screening the transformants are indicated by arrows.

b. PCR agarose gel picture of the LR recombination test

Lane 1	3'element plasmid	***Lane 6***	clone 4	***Lane 11***	clone 3
Lane 2	p R225(*hph* gene cassette)	***Lane 7***	clone 5	***Lane 12***	clone 2
Lane 3	clone 1	***Lane 8***	1 Kb+ ladder	***Lane 13***	clone 1
Lane 4	clone 2	***Lane 9***	clone 5	***Lane 14***	5'element plasmid
Lane 5	clone 3	***Lane10***	clone 4	***Lane 15***	pR225(hph gene cassette)

Controls for PCR reactions included a no-template DNA control as well as negative controls containing individual 3' or 5' element donor vectors or the *hph* cassette. The 5 randomly picked clones (clones 1-5) gave the PCR products of the expected size (0.9 kb) with these two pairs of checking primers. Clone 1 was selected for further use as

disruption vector pR246. The junctions between the 5'element and *hph* gene and the 3' element and *hph* gene were sequenced (sequence in Appendix III). All these negative controls should not have PCR products. However, as shown in Fig 4.2b, in lane 14, the 5'element plasmid also had a small PCR product (~ 0.4 kb) with primer pair *epoAKO1* and *5'hphout,* which is smaller than the positive band (~ 0.9 kb). This band is probably a non-specific binding product (see discussion).

4.2.4 Factors affecting transformation efficiency

The 3'element BP recombination and LR recombination reactions were all cleaned with PCR column. There were 12 transformants with the PCR column cleaned 3'element BP recombination compared to no transformants from the proteinase K treated 3' element BP reaction. The PCR column cleaned LR reaction had hundreds of transformants. In order to find out whether the greater transformation efficiency was caused by using the PCR purification column instead of proteinase K treatment, a 20 µl LR reaction system was set up as described in Section 2.4.3.2.

Sixty ng of each of the vectors were used in the reaction. Then the reaction mixture was spilt up for different treatments prior to transformation. A 5 µl (15 ng) reaction mixture was treated with proteinase K. A 10 µl volume (30 ng) of the reaction mixture was cleaned by PCR column and eluted in 30 µl volume; the concentration of the vector is approximate 1 ng/µl. One µl (~ 3 ng vector DNA) of each of the proteinase K treated and untreated LR reaction mixture was transformed by both electroporation and chemical transformation. In addition, 2 µl (~ 2ng vector DNA) PCR column cleaned DNA was used for electroporation and chemical transformation. The comparison of the transformation results is shown in Table 4.2; expressed as transformants per nanogram of DNA. The results show that the PCR column (QIAGEN) cleaned DNA had greater transformation efficiency than the non-cleaned reaction DNA.

Table 4.2 Comparison of the transformation results (transformants /ng vector DNA)

Transformation method	PCR Column cleaned	Without PCR column cleaned		Negative control
	NO proteinase K	No proteinaseK	proteinase K	
Electroporation	392	164	36	0
Chemical	54	24	19	0

Furthermore, electroporation transformation had a greater efficiency than chemical transformation, yielding more transformants. Finally, in this trial, the use of proteinase K (recommended by the Gateway ™ manufactures) did not increase transformation efficiency.

4.3 TARGETED DELETION OF THE PUTATIVE EPOA GENE IN *D. SEPTOSPORUM*

To disrupt the putative *epoA* gene, the disruption vector pR246 was transformed into *D. septosporum* wild type (Section 2.5.2). Two new field isolates of *D. septosporum,* NZE7 and NZE8 were used for the transformation due to their high dothistromin production. A double crossover by homologous recombination would result in replacement of a portion of the *epoA* gene (the coding region between primers *epoAKO2* and *epoAKO3*) with the *hph* cassette.

After one week's incubation at 22°C, numerous tiny colonies (< 2 mm) were observed on the hygromycin B containing plates. However, only a few of them continued growing through the top agar to a size of approximately 4-10 mm about 2 weeks after the transformation. These colonies were sub-cultured onto DM plates containing 70 mg/L hygromycin B.

Stable colonies regenerated from sub-culturing were recorded as true transformants and the number used to calculate the transformation frequency. In contrast, protoplasts treated the same but without DNA were plated onto non-selective media to calculate the regeneration frequency. The protoplast regeneration efficiency was estimated to be 1%. The transformation results are shown in Table 4.3.

Table 4.3 Transformation results of pR226

Plasmid (5 µg circular)	*D. septosporum* strains	Total transformants	Transformation frequency (μg^{-1})
pAN7-1 (1µg / µl)	NZE7	424	84.8
pR246 (60 ng / µl)	NZE7	40	8.0
pR246 (150 ng / µl)	NZE8	102	20.4

The vector pAN7-1 was used as a positive transformation control and untransformed protoplasts on hygromicin B plates were used as a negative control. The positive control transformation had 424 transformants per 5 μg DNA. In contrast, no transformants grew on the negative control plates as expected. The transformation frequency was calculated as transformants per microgram DNA.

4.3.1 Pre-screening for *epoA* deletion mutants by PCR

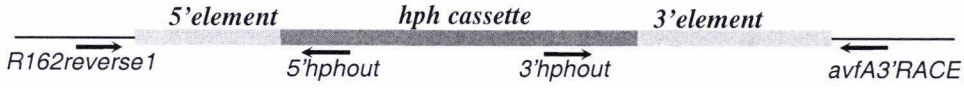
The stable transformants were initially screened by PCR. Primers for PCR amplification (Section 2.6.2) were designed to identify homologous recombination events and the deletion of the *epoA* gene as shown in Fig 4.3a Primers *R162reverse1* (flanking the 5'element region) and *5'hphout* (in *hph* gene region) were used to detect homologous recombination in the 5' region. A 1.3 kb PCR product was an indication of homologous recombination in this region. Primers *avf3'RACE* (flanking the 3'element region) and *3'hphout* (in *hph* gene region) were used to detect homologous recombination in the 3' region. A 1.1 kb PCR product was an indication of homologous recombination in this region. The primers' positions are indicated in Fig 4.3a. Only when homologous recombination occurred at the 5 'and 3' ends, could products be obtained from both 5' and 3' region PCR.

PCR screening commenced with NZE8 transformants. Genomic DNA of all the 102 NZE8 transformants was extracted (Section 2.3.1) and screened by PCR twice. Fig 4.3b shows the first round PCR screening; there were only three *epoA* mutants (E18, E19 and E21) in 102 transformants. However, the PCR did not work very well with the DNA templates. The DNA of all the samples was subsequently purified and different dilutions of the DNA samples were used as templates for PCR. It was found that 10 fold dilutions of the cleaned template DNA gave good PCR results. Most of the transformants were screened again with diluted DNA templates. Another two transformants, E4 and E6, were shown to be mutants. Results for some of these are shown in Fig 4.3c. At this stage, it is not certain which transformant is a double crossover and which is single crossover. The results may different after single spore purification.

As mentioned in the introduction, protoplast transformants are normally heterokaryons. In order to obtain homokaryons, two rounds of single-spore isolation (Section 2.2.2.1)

Fig 4.3 Preliminary screening of *epoA* transformants

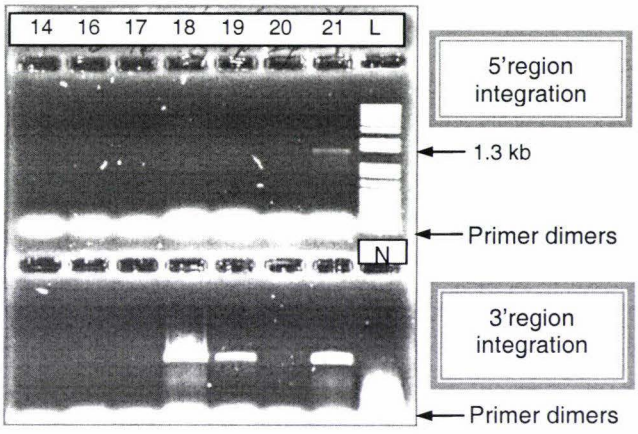
a. Schematic map of preliminary screen of *epoA* transformants



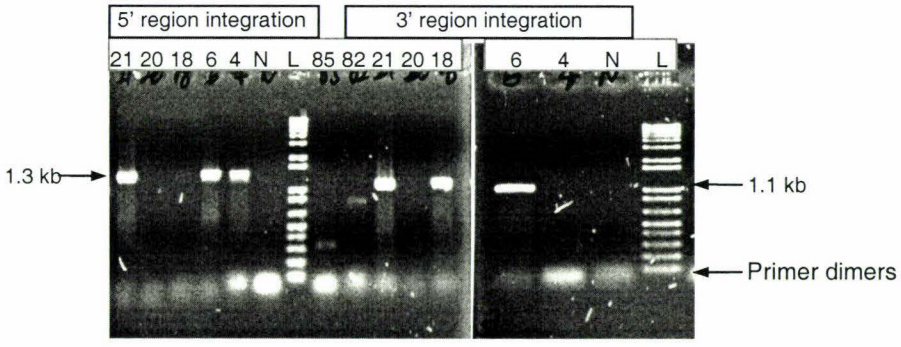
Primers' positions for screening the transformants were indicated by arrows.

——: Genomic DNA

b. PCR results of the first round pre-screening of *epoA* transformants



C. PCR results of the diluted DNA templates of *epoA* transformants



The numbers are transformant colony numbers that indicate their names.

N: negative control (the same PCR mixture without template)

L: 1 kb^{+} Ladder

The *epoA* 5' and 3' integration PCR products are between 1 kb and 1.65 kb. Because the first gel did not have a ladder for 3'region PCR products, PCR of E18 and E21 were repeated to test if the products size is right. From these two gels results: E21, E6 and E4 have 5'region PCR products; E19, E21, E18 and E6 have 3'region PCR products. The small bands (< 100 bp) are primer dimers.

were carried out on all the five putative mutants E4, E6, E18, E19, E21 and three randomly selected ectopic transformants E1, E10 and E11, which were used as ectopic controls. DNA of the substrains generated from the eight selected transformants were prepared (Section 2.3.2). Then the genomic DNA of all the single spore purified transformants was checked by PCR again. E19 and E21 were shown to be double crossover mutants. So, Southern hybridization was carried out with E19 and E21, using WT NZE8 and ectopic transformant E10 as controls.

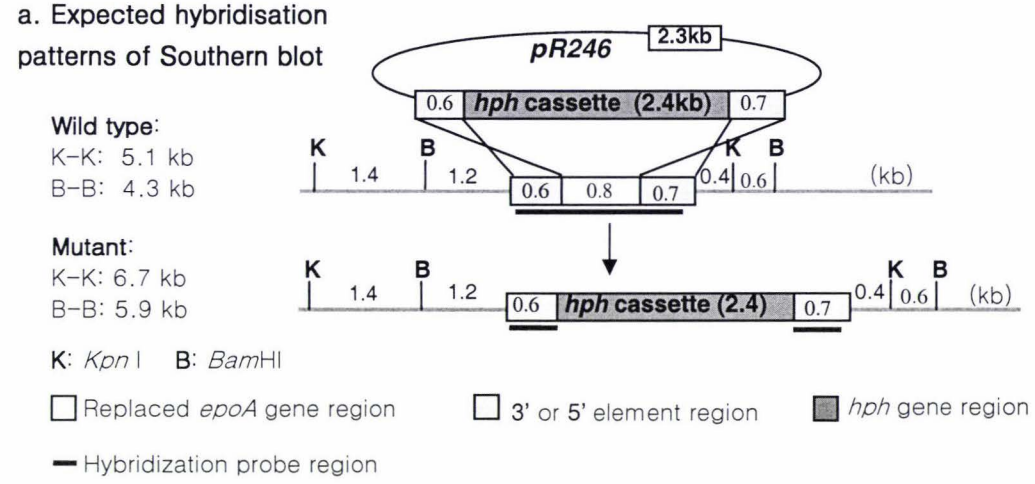
4.3.2 Southern blot hybridization analysis

Southern blot hybridisations (Section 2.8) were employed to confirm deletion of the *epoA* gene, to examine the copy number and the site of recombination of the replacement construct into the genome. The hybridization probe was a 1.9 kb PCR product from NZE8 genomic DNA, amplified with primers *R162reverse2* and *R162forward2* (positions shown in Fig 4.4a). *KpnI* and *BamHI* digestions were used for confirming the mutants. As expected, in a double crossover event, a 0.8 kb *epoA* gene fragment should be replaced by a 2.4 kb *hph* gene cassette. Since there are no *BamHI* or *KpnI* sites on the disruption vector pR246, the mutants should give a 1.6 kb bigger fragment than that in WT. As shown in Fig 4.4a, a 5.1-kb *KpnI* and a 4.3 kb *BamHI* fragment should hybridize in wild-type but a 6.7 kb and a 5.9 kb fragment respectively in double crossover gene replacement mutants. The expected mutant pattern of hybridiztion was seen in transformants E19 and E21 (Fig. 4.4b, lanes 4-7).

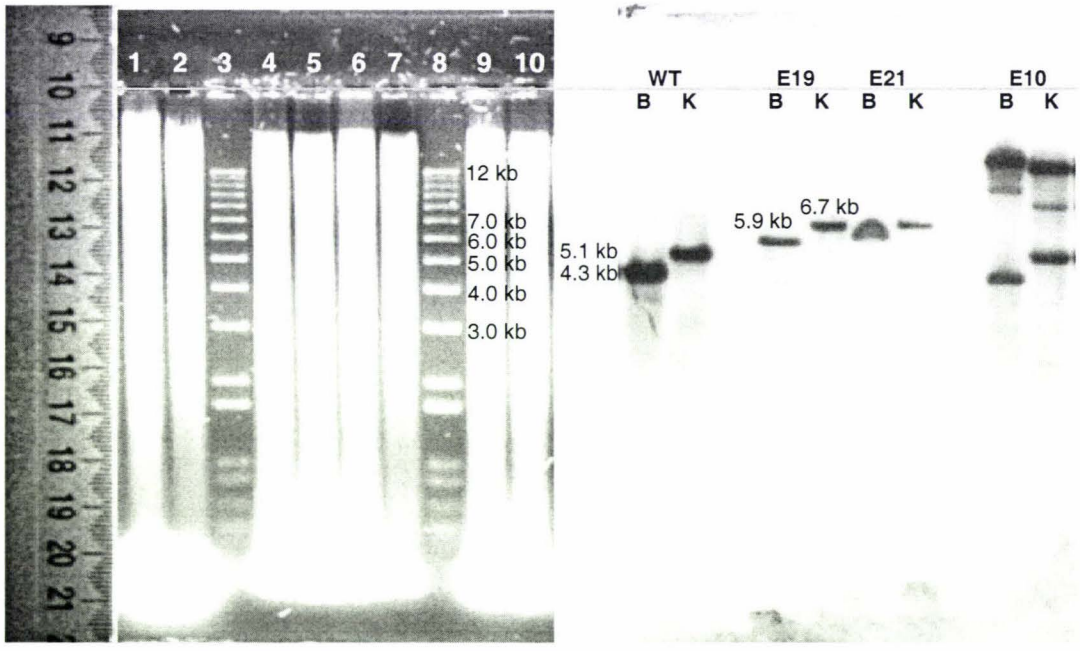
However, for mutant E21, although the *KpnI* digestion gave an expected size band, the *BamHI* result was not very clear, possibly due to a gel running problem. From the Southern blot gel picture, the DNA in lane 6 was more smeared than the lane 7 and suggested overloading and /or impurity in the DNA sample. The ectopic strain E10 (Fig 4.4b, lanes 9 and 10) had a same size band as the wild type (a 4.3 kb */BamHI* band and a 5.1 kb */KpnI* band) and two extra ectopic bands that did not appear in wild type results. Therefore, the Southern hybridization results confirmed that both E19 and E21 were *epoA* gene double crossover mutants, whilst E10 is an ectopic strain.

Fig 4.4 Southern hybridization to confirm the identified *epoA* mutants

a. Expected hybridisation patterns of Southern blot



b. Southern blot gel (Left) and Southern hybridization X-ray film (Right)



Lane 1	NZE8/BamI	**Lane 2**	NZE8/KpnI	**Lane 3**	1 kb+ ladder
Lane 4	E19/BamH I	**Lane 5**	E19/KpnI	**Lane 6**	E21/BamH I
Lane 7	E21/KpnI	**Lane 8**	1 kb+ ladder	**Lane 9**	E10/BamH I
Lane 10	E10/KpnI				

NZE8 is a wild type strain. E19 and E21 are epoA mutants, E10 is ectopic.
The hybridization products sizes are marked above or alongside the band.
Some 1kb+ fragments sizes are indicated under the bands.

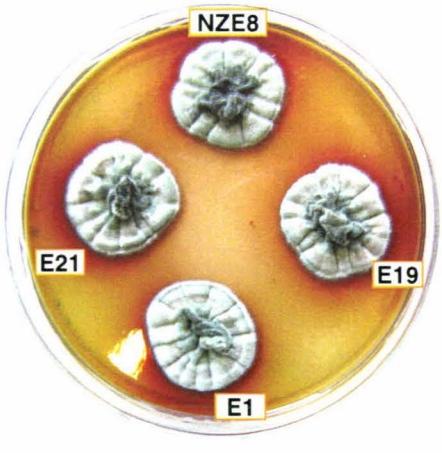
4.4 CHARACTERIZATION OF THE *EPOA* MUTANTS

By comparing the phenotypes of the *epoA* mutants and the wild type, it can be determined whether *epoA* is involved in dothistromin biosynthesis. TLC was used to check the accumulation of dothistromin in the mutants and competitive ELISA was employed to quantify the dothistromin production of the mutants. Growth and sporulation rates were compared to check if the gene mutation has any effect on these parameters. Finally, the disrupted *epoA* gene expression was checked by northern hybridization.

4.4.1 TLC analysis of the *epoA* mutant

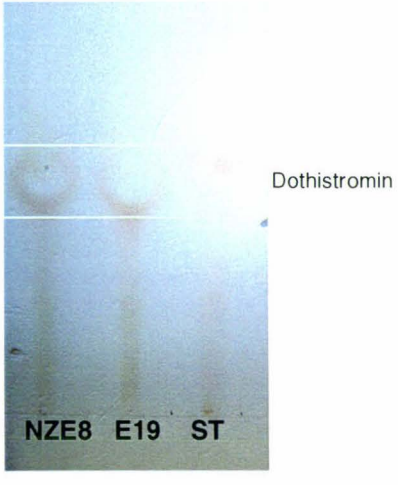
The *epoA* mutants' phenotype has no immediately apparent differences from the wild type when grown on selective DM plates, as shown in Fig 4.5a. The mutants still produce the dark red pigment as wild type does. The mutant E19 was used to test for the dothistromin production and WT NZE8 was used as a positive control of the test. The intermediates were extracted and run on a TLC plate (Section 2.12). As shown in Fig 4.5b, there is no clear difference in metabolites production from the TLC plate. E19 also has a red colour band that indicates dothistromin production.

Fig 4.5 TLC results of the *epoA* mutant intermediates



a. mutants phenotype

E21, E19: *epoA* mutants
E1: ectopic transformant
NZE8: wild type



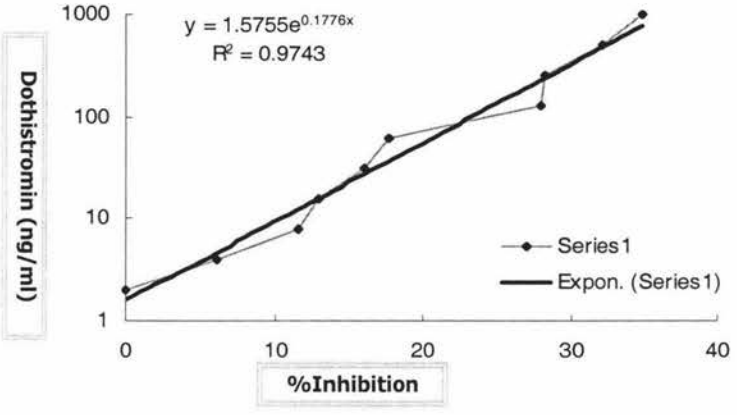
b. TLC to check *ΔepoA* intermediates

ST: dothistromin standard

4.4.2 ELISA analysis of the *epoA* mutants

From the TLC result, the *epoA* mutant appears to produce dothistromin, but how much it produces and whether the dothistromin production of the mutant is less than wild type is still not clear. Competitive ELISA was carried out to quantify the production of dothistromin (Section 2.15).

Fig 4.6 The comparison of dothistromin production between *epoA* mutants and WT



Dothistromin standard

dothistromin (ng/ml)	Inhibition
1.95	0
3.9	6.0
7.8	11.6
15.6	13.0
31.2	16.0
62.5	17.7
125	28.0
250	28.3
500	32.2
1000	34.9

* dothistromin standard curve data are shown in the right side table.
The correlation coefficient, **R** value, is a number between 0 to 1, the R value closer to 1, the closer between the predicted values and the actual values.

The dothistromin production of the *epoA* mutants

Strain	%Inhibition#	*Dothistromin (ng/ml media)	Dry weight (g)	*Dothistromin (ng/mg DW)
E19-1	31.3	4086.6	0.19	322.6
E19-2	34.9	7744.6	0.27	430.3
E19-3	34.1	6718.9	0.22	458.1
E21-1	32.6	5147.7	0.29	249.1
E21-2	28.0	2274.3	0.31	117.6
E21-3	35.0	7883.4	0.34	347.8
E6-1	22.1	797.7	0.29	54.4
E6-2	24.8	1288.4	0.22	69.0
E6-3	31.0	3874.6	0.28	200.4
E11-1	33.8	6370.4	0.1	955.6
E11-2	29.9	3302.3	0.06	825.6
E11-3	30.1	3187.0	0.06	796.8
NZE8-1	29.8	3130.9	0.16	293.5
NZE8-2	31.4	4159.8	0.27	231.1
NZE8-3	31.0	3874.6	0.22	264.2

#: the inhibition is from the 10 fold dilution of each sample.
* The dothistromin concentration was calculated with the formula: $y = 10 \times 1.5755e^{0.1776x}$, **10** is the dilution fold.

In order to have a reliable result, besides mutants E19 and E21, the dothistromin production of another mutant E6 was also analyzed in this assay. One ectopic strain E11 and the wild type NZE8 were analyzed as controls. The pure dothistromin standard was used to prepare a stand curve for the quantification as before. The results are also shown in Fig 4.6.

The dothistromin production of mutants and the wild type was analyzed by an unpaired t-test (http://www.graphpad.com). The results are shown in Table 4.4. The dothistromin production of mutants E21 was not statistically different from the wild type. However, of the other two mutants, E19 showed significantly higher dothistromin production but E6 showed less dothistromin production compared to WT. This result demonstrated that the dothistromin production was variable in different mutants and that overall there is not a difference between the *epoA* mutants and the wild type strain. In contrast, the ectopic strain E11 produced much more dothistromin than the WT.

Table 4.4 Unpaired T-test of dothistromin production of the *epoA* mutants

Strains	Dothistromin production Mean ± SD (ng/mg DW)	T#	p
E19 (*epoA* mutant)	403.7 ± 71.6	3.1222	*0.0355
E21 (*epoA* mutant)	238.2 ± 115.5	0.3585	0.7381
E6 (epoA mutant)	107.8 ± 80.2	3.1198	*0.0355
E11 (ectopic)	859.3 ± 84.6	11.4584	*0.0003
NZE8 (wild type)	262.9 ± 31.2	-	-

Each mutant strain was compared with NZE8 wild type.
* indicates a statistically significant different from WT.

4.4.3 Growth rate of *epoA* mutants

To test whether the *epoA* mutation has an effect on the growth of *D. septosporum*, the radial growth rates of the mutants and WT were measured and compared as described in Section 2.16. Three mutants (E19, E21 and E6), two ectopic transformants (E1 and E11), and WT NZE8 were tested. Colonies were grown on DM plates at 22°C and radial growth measured at 5, 10, 15, 23 and 28 days. Each sample had 9 replicates. Radial growth data are shown in Table 4.5.

Table 4.5 Radial Growth of *epoA* mutants

Strain	5 days	10 days	15 days	23 days	28 days
	Mean±SD (mm)	Mean±SD (mm)	Mean±SD (mm)	Mean±SD (mm)	Mean±SD (mm)
[a] **NZE8** (wild type)	4.8 ± 0.35	9.8 ± 0.77	13.9 ± 0.70	20.7 ± 0.71	23.7 ± 1.10
[a] **E21** (double cross-over)	5.0 ± 0.35	9.8 ± 0.73	14.2 ± 0.98	20.2 ± 0.89	23.2 ± 1.13
[a] **E19** (double cross-over)	4.8 ± 0.38	9.9 ± 0.48	14.2 ± 0.54	20.8 ± 1.09	23.5 ± 0.76
[a] **E1**(ectopic)	4.8 ± 0.30	9.8 ± 0.66	14.3 ± 0.47	20.9 ± 0.84	23.7 ± 0.71
[b] **NZE8**(wild type)	5.1 ± 0.26	10.2 ± 0.43	14.1 ± 0.68	20.61 ± .93	24.8 ± 0.92
[b] **E6** (3'single cross-over)	5.1 ± 0.47	10.8 ± 0.82	15.1 ± 0.75	21.7 ± 1.49	25.8 ± 1.41
[b] **E11**(ectopic)	4.9 ± 0.28	*9.1 ± 0.70	*11.6 ± 0.79	*16.8 ± 0.49	*19.3 ± 0.73

n = 9; nine replicates of each tested strain.

a and **b** indicated two sets of plates, the strains labeled "a" are one plate sets; the strains labeled "b" are another plate sets. The tested mutant strains were compared to the wild type strain in their sets, respectively.

*: indicates the data statistically significant, when comparing mutant to wild type within each set.

The ectopic strain E11 grew significantly ($p<0.05$) slower than WT from day 10 to day 28. There is no difference between the other strains and WT. Subsequently, the growth rate for each strain was calculated to allow an overall comparison over the growth period, the data are shown in Appendix Table 3. The growth rate was calculated as the average of the growth per day over 23 days (5-28 day). The ectopic strain E11 showed extremely slow growth (p=0.0001) compared to the WT, although the other strains including the ectopic strain E1, showed similar growth rates to WT. Hence, the *epoA* mutation has no effect on the colony growth rate.

4.4.4 Sporulation of *epoA* mutants

In order to test whether the *epoA* gene mutation affects sporulation rate, the sporulation of two *epoA* mutants (E21 and E19) and WT NZE8 were compared (Section 2.17). Three replicates of each strain were checked.

The results were analyzed by an unpaired t-test (http://www.graphpad.com) and are shown in Table 4.6. There is no significant difference between the mutants and WT. The results indicate that the *epoA* mutation has no effect on the sporulation rate.

Table 4.6 Sporulation of the *epoA* mutants

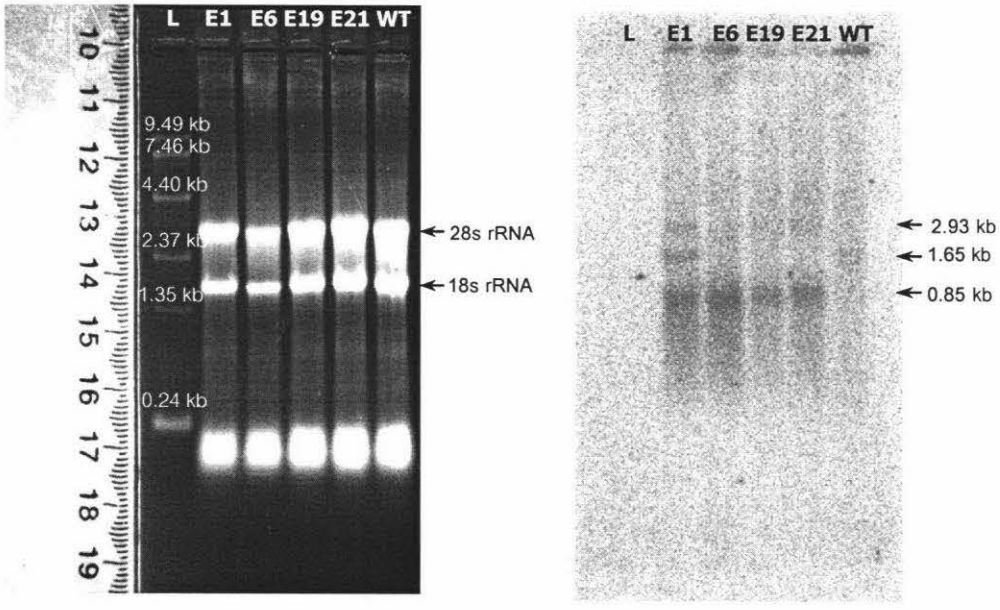
Strain	Spores×10^5 per ml	Unpaired *T-test* of *epoA* mutant/WT	
	Mean ± SD	*T*	*p*
E19 (double cross-over)	30.83 ± 8.96	0.6005	0.5805
E21 (double cross-over)	22.00 ± 20.62	0.4370	0.6847
NZE8 (wild type)	27.33 ± 4.64	-	-

n=3, three replicates of each tested samples.

4.4.5 Northern hybridization

Both TLC and ELISA results showed the *epoA* mutants produced dothistromin. Whether the *epoA* mutant still expresses functional mRNA was tested by northern hybridization (Fig 4.7). Ectopic strain E1 and WT NZE8 RNA were used as a control for the hybridization. Three mutants, E6, E21 and E19, RNA were tested. The probe was the one used for Southern hybridization to identify the mutants (Fig 4.4a).

Fig 4.7 Northern hybridization results



L: RNA ladder
E1: ectopic strain
WT: wild type NZE8
E6: *epoA* mutant
E19: *epoA* mutant
E21: *epoA* mutant

Clear and sharp 18s and 28s rRNA band in the gel indicated the quality of the RNA samples.

The northern hybridization results (Fig 4.7) demonstrate that the ectopic strain E1 has a band the same size as wild type, and one small band as mutants and an extra ectopic band. All three mutants express smaller mRNA than the wild type. From the gel, the difference of the size between mutants and WT *epoA* mRNA is approximated 0.8 kb, which is equivalent to the size of the replaced region.

4.5 DISCUSSION

EpoA gene mutants were obtained by gene disruption. The mutants were characterized by four different assays, such as TLC, ELISA, growth rate and sporulation rate. However, none of them could distinguish the *epoA* gene function.

4.5.1 Construction of the *epoA* disruption vector

The Gateway system had been used for construction of the *pksA* disruption vector (Teddy, 2004) and was also used in this work to construct the *epoA* disruption vector. In Teddy's research, she found that the purification method recommended by the manufacturer was not successful and that recombination between circular DNA and donor vector was easier than between linear DNA and donor vector. Therefore, in this research, the PCR products tailed with *attB* sequence were purified with a PCR purification kit and then cloned into pGEMT or pGEM-T Easy vector to create a circular DNA vector. The BP and LR recombination reactions incubation time (overnight) was according to previous research too (Teddy, 2004) instead of the time recommended by the manual.

The LR recombination transformation efficiency of this study was much greater than Teddy's results. The same amount of the pDEST vector (60 ng) was used in both studies. Eight transformants of the LR recombination were obtained in previous study (Teddy, 2004), whilst hundreds of transformants were generated in this study. The only difference was that the LR recombination mixture was purified by PCR column prior to transformation.

In order to test if the high transformation efficiency is caused by the purification, LR recombination was repeated, treated with either proteinase K or PCR column and then

transformed into two different types of competent cells (electroporation and chemical competent cells). The comparison of the transformation efficiency between the PCR column clean and the recommended treatment with proteinase K demonstrated clearly that the PCR column was better than the recommended method (Table 4.2). In this study, electoporation transformation had higher transformation efficiency than chemical transformation whereas in the previous study, chemical transformation efficiency was greater than electroporation transformation. It is not sure if the low transformation efficiency of electroporation was caused by too much DNA (300 ng vector DNA compare to 2ng vector DNA used in this study) used for the transformation in the previous study (Teddy, 2004).

In the screening PCR of the LR recombination transformants, there is a small PCR product from the 5'element plasmid (lane 14 in Fig 4.2b, there should be no product). A similar size band was also obtained from the BP recombination screening, using 5'donor vector pDONR P4-P1R as a template. The PCR was repeated with p5'element and pDONR P4-P1R as templates to find out what causes the products. However, there was no product from either PCRs with the two different plasmid templates. It is suggested that the PCR product from the LR recombination screening was a non-specific binding product.

4.5.2 Transformation of *D. septosporum* protoplasts

Transformation of the *epoA* disruption vector into *D. septosporum* was performed using the same procedure as transformation of *pksA* disruption vector. The transforming DNA concentration also appears to have an effect on the transformation frequency as shown in Table 4.3 and as seen with *pksA*. In NZE8 transformation, the transformed vector DNA concentration was 150 ng/µl, while it was 60 ng/µl in NZE7 transformation, whilst the transformation frequencies of these were 20.4 and 8.0, respectively.

Only five *epoA* mutants were identified (Fig 4.3 b and c) in 102 transformants from NZE8 transformation. This may be caused by the shorter homologous regions (0.6 kb and 0.7 kb) used for constructing the disruption vector, because for *pksA* transformation, 8 mutants were obtained from 50 transformants. It was also reported that the longer

homologous region used in the disruption vector, the more mutants could be obtained (Bird and Bradshaw, 1997).

4.5.3 Identification of the *epoA* mutants

The *epoA* transformants were preliminarily screened by PCR and the putative *epoA* mutants were purified by two round single spore purification as for the *pksA* mutants. The purified mutants were analysed by Southern hybridization with *KpnI* and *BamHI* digestions. The Southern hybridization results of the mutants E19 and E21, WT and ectopic strain E10 were as expected (Fig 4.4 b). The ectopic strain had a WT size band and other two large size bands. In the mutant E21's *BamHI* digestion (Fig 4.4 b lane 6), the hybridized band was not straight as the other bands, this problem might be caused during the gel running. However, the *KpnI* digestion of the E21 hybridized band was clear, so E21 is a double crossover mutant. Of the 5 putative mutants, two of them, E19 and E21, were confirmed to be double crossover mutants.

4.5.4 Characterization of the *epoA* mutants

There was no difference in appearance between the confirmed mutants and the wild type NZE8 grown on a DM plate (Fig 4.5a). The mutants still produced the dark red halo of dothistromin (Fig 4.5a). The *epoA* mutants were analysed by four assays as for *pksA* mutants. Intermediates of mutant E19 were checked by TLC assay (Fig 4.5b); the mutant had a similar result to WT. It was thought that *epoA* may be involved in a very late step of dothistromin biosynthesis and the TLC could not distinguish the products of the late steps. Hence, competitive ELISA was used to quantify the production of the dothistromin.

Competitive ELISA was reported as a reliable method that could detect dothistromin with a specific antibody. ELISA was used successfully in the characterization of *dotA*. However, in this study, it was found that the antibody used in ELISA was not unique to dothistromin and it interacted strongly with some intermediates, such as the intermediates AVR and AVTH used in *pksA* mutant feeding assays. Therefore, although

the *epoA* mutants appeared to produce dothistromin on the ELISA assay, it is possible that a related precursor was being detected instead. Both ELISA and TLC were not suitable to fully characterize the *epoA* mutants.

Moreover, both growth rate (Table 4.5) and sporulation rate (Table 4.6) showed no difference between the mutants and the WT. The sporulation rate result was expected, because there was no difference in dothistromin production of the mutants compared to wild type. On the other hand, it also supported the idea that sporulation rate is associated with the secondary metabolites. The ectopic strain E11 had slower growth compared to the WT (Table 4.5) and it had higher dothistromin production (Fig 4.6). This result may be caused by the ectopic integration in the genome; the transformed vector may have integrated at a regulatory gene region and affected dothstromin production.

The function of *epoA* was still not clear after these four assays. Southern hybridization confirmed that the targeted 800 bp *epoA* coding region was replaced by a 2.4 kb *hph* cassette on the mutant genome. Genetically, the gene, *epoA,* was disrupted. In order to test the expression of the disrupted gene, northern hybridization was performed. The intensity of RNA was indicated by 18s and 28s rRNA (Fig 4.7). The hybridized bands were excluded from being 18s and 28s rRNA due to the facts that they were different sizes and that the tested samples had different size bands to each other.

The mutants had a smaller hybridized band (0.85 kb) compared to the wild type (1.65 kb) confirming that *epoA* was disrupted. It is feasible that the *hph* cassette was spliced out and the gene mRNA fragments were connected. This result could be caused by the two *attB* sites of the construct. In the *attB* sequence flanking the 5' *hph* cassette, there is a 6 bp oligo sequence GTTTGT that matches the fungal consensus 5' intron splice site (GT(A/G/T)NGTY) whilst there is 3 bp - CAG (3'YAG intron splice site) in the *attB* sequence flanking the 3'*hph* cassette. Therefore, the *hph* cassette may be recognized as a big intron and spliced during the expression. However, the ectopic strain had three different bands, including a 0.85 kb, a 1.65kb (WT) and a 2.93 kb. The 1.65 kb band as WT could be explained that it was from *epoA* expression, but in this case, the origins of the small band (0.85 kb) and the 2.93 kb band are unclear.

4.5.5 The possible functions of *epoA*

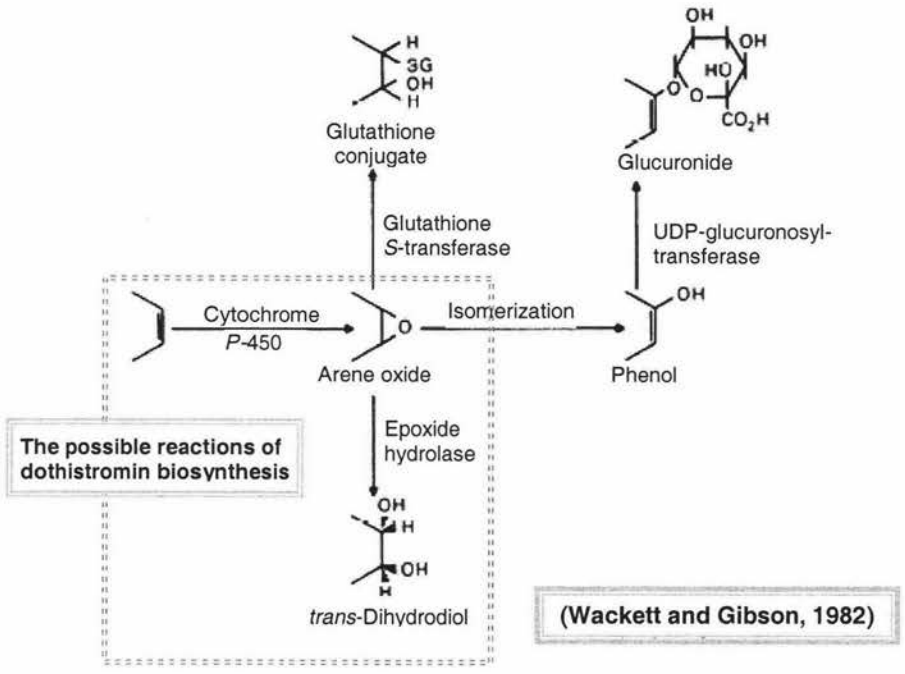
The *epoA* ORF is 1314 bp in length including one 51-bp intron and corresponding to a polypeptide of 438 amino acids. The deduced amino acid sequence showed high similarity to that of EH in *A. niger* (Teddy, 2004) and it belongs to cluster III microsomal EHs (Barth *et al.*, 2004). The EH in *A. niger* contained the same catalytic triad (Asp^{192}, Asp^{348} and His^{374}) as in mammalian EHs. Compared to EH in *A. niger*, the deduced EpoA in *D. septosporum* has Asp^{197} (Asp^{192} in *A. niger*) and His^{372} (His^{374} in *A. niger*) two active site residues, but Thr^{346} instead of Asp^{348} in *A. niger*. These three residues were characterized by site-directed mutagenesis in *A. niger*, which showed that mutation of any of them resulted in loss of enzyme function (Arand *et al.*, 1999).

However, in a study of dothistromin gene expression by RT-PCR, the *epoA* gene has the same expression pattern as *pksA* and *dotA* genes (Schwelm, A. personal communication). The gene expression of *epoA* reaches a peak in 3 and 4 days mycelia and declines in 12 days mycelia. The northern hybridization in this study also showed that *epoA* mRNA was expressed at 6 day's culture of the wild type. Given that the EpoA is a putative mEH, the mEHs belong to the α/β hydrolase fold and catalyze hydrolysis of epoxides to the corresponding diols. Some of them are involved in the synthesis of secondary metabolites. In a fungus *Beauveria densa*, EH production is concomitant with a secondary pigment produced in a stationary phase or idiophase (Grogan *et al.*, 1996).

The study of the production of *Alternaria alternata* f. sp. *lycopersici* host-specific toxin (AAL toxin) indicated that the EH activity is associated with AAL toxin production (Morisseau *et al.*, 1999). The study of metabolism of phenanthrene by the fungus *Pleurotus ostrestus* suggested that phenanthrene was oxidized by a cytochrome P-450 monooxygenase and then followed by an epoxide hydrolase catalyzed hydration reaction (Bezalel *et al.*, 1996). The oxidation of aromatic hydrocarbons by the filamentous fungus *Cunninghamella elegans* showed remarkable similarity to mammals (Wackett and Gibson, 1982). The reactions used by mammals for the metabolism of aromatic hydrocarbons are shown in Fig 4.8.

There is a putative cytochrome P-450 monooxygenase gene (*cypA*) and a monooxygenase gene (*moxA*) in the identified dothistromin gene cluster. The

Fig 4.8 The reactions of the metabolism of aromatic hydrocarbons by mammal

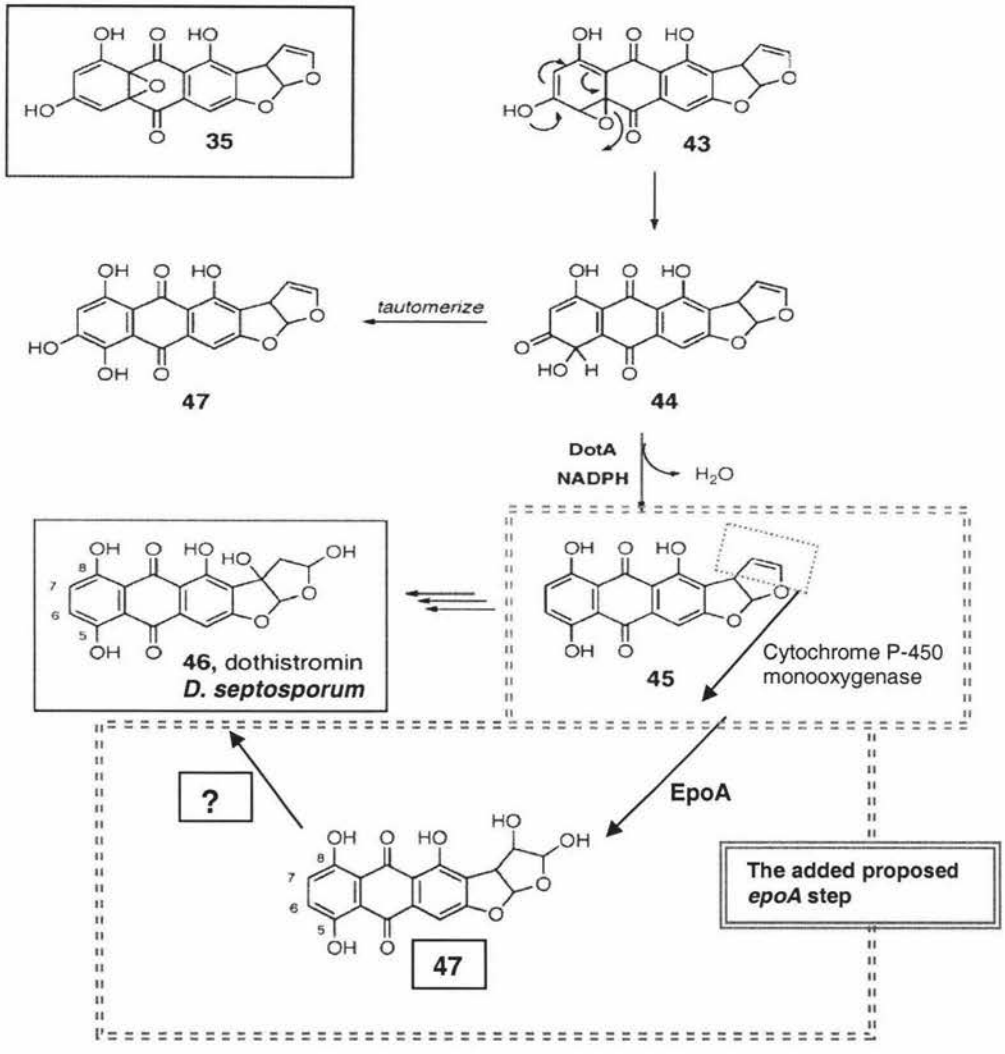


cytochrome P- 450 oxidative steps in AF biosynthesis and related fungal metabolites have been studied recently (Henry and Townsend, 2005). Because the existence of VERA and 5, 8- dihydroxy anthraquinone (44, in Fig 4.9) in *D. septosporum*, they proposed that the A-ring of VERA (43 in Fig 4.9) in dothistromin biosynthesis in *Dothistroma* was different from that in AF biosynthesis (35 in Fig 4.9) in *Aspergillus*. They assumed that VERA was rearranged to form an A ring edge epoxide (43 in Fig 4.9), then gave the secondary alcohol 44 (Henry and Townsend, 2005), a step that could involve an epoxide hydrolase enzyme.

However, the *epoA* gene disruption results indicated that if the *epoA* gene is involved in dothistromin biosynthesis, it should be in a later step than that of the *dotA* gene, as *dotA* mutants were impaired in dothistromin biosynthesis whilst *epoA* mutants were not. Therefore, in dothistromin biosynthesis, aromatic hydrocarbons of 45 (in Fig 4.9) may be oxidized by a monooxygenase and then hydrolyzed by epoxide hydrolase as in *P. ostrestus* (Fig 4.8) to form an intermediate (47, Fig 4.9). If this hypothesis is right, the structure of the EpoA precursor 47 is likely to be very similar to dothistromin 46. The TLC, ELISA, growth rate and sporulation rate assays cannot characterize the function of the gene. A specific dothistromin detection method will be required to distinguish

dothistromin and its close precursors. HPLC and NMR analysis have been used to detect DHST accumulation in *stcL* mutant, because it is hard to distinguish DHST and ST on TLC plates (Keller and Hohn, 1997). Therefore, these two methods may also be suitable for analysing the accumulation in *epoA* mutants.

Fig 4.9 The proposed dothistromin biosynthetic steps (Henry and Townsend, 2005).



However, the lack of one active amino acid corresponding to Asp^{348} in *A. niger* raises a problem that the EpoA in *D. septosporum* may be a non-functional enzyme. Therefore, the EpoA activity of the WT needs to be checked. Although, both Southern and northern hybridization confirmed that *epoA* was disrupted, the EpoA activity needs to be checked to confirm that not only there is no active epoxide hydrolase in the mutants, but also that there is active epoxide hydrolase in the wild type.

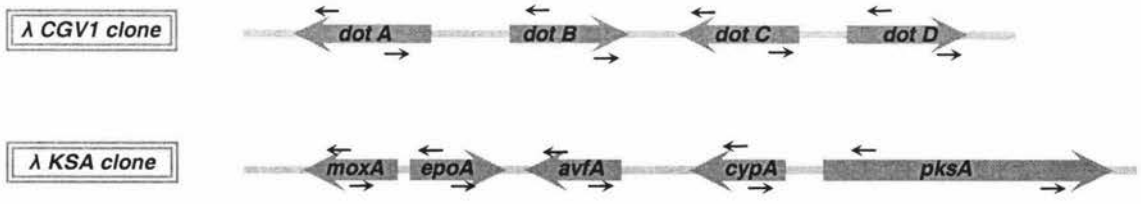
CHAPTER FIVE: RACE RESULTS

5.1 RACE ANALYSIS

Rapid Amplification of cDNA ends (RACE) provides a method to obtain full-length 5' and 3' ends of cDNA and to identify and characterize the untranslated mRNA region (UTR). This was achieved by adding two RNA oligos to both the 5'and 3' ends of the mRNA, then using the primers designed to these two RNA oligo regions, along with gene specific primers designed to each targeted gene exon region, to amplify the 5'and 3' UTRs. RACE was carried out as described in Section 2.9. The nine putative dothistromin genes were all analyzed by RACE. They were divided into two groups (Fig 5.1). One group is the genes on the λCGV1 clone, *dotA, dotB, dotC* and *dotD* that have been published and relatively well characterized (Bradshaw *et al.*, 2002). Another group is the genes on the λKSA clone, *pksA, cypA, avfA, epoA,* and *moxA,* which have been identified recently.

Fig 5.1 The identified dothistromin genes

The small black arrows indicate positions of gene specific primers used for RACE.



5.2 RNA PURIFICATION AFTER EACH REACTION OF RACE PROCEDURE

The manufacturers' instructions for the RACE kit recommended purification of RNA with phenol/chloroform to remove enzyme prior to the next reaction. However, the RNA was purified by PCR column instead of phenol/chloroform because a PCR column was expected to clean up the protein from the reaction system more effectively and it is both easier and less hazardous than the recommended method. DEPC-H_2O was used for eluting RNA instead of the elution buffer from the kit. Whether the PCR column was suitable for RNA purification was checked by running the purified samples on a formaldehyde gel. Aliquots of the purified RNA from dephosphorylation and decapping reactions were checked as shown in Fig 5.2. The RNA integrity was indicated by the two sharp bands of 28s and 18s ribosomal RNA (rRNA). The RNA was still good. The

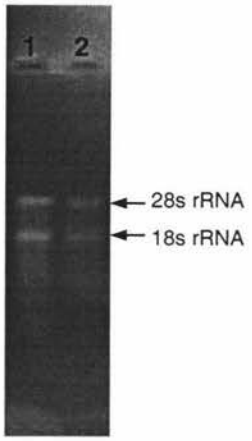
purified dephosphorylated RNA was used for the de-capping reaction, the comparison of brightness of the two purified RNA sample bands from the dephosphorylation and decapping reactions was used to estimate the recovery efficiency, which was acceptable. The reverse transcribed first strand cDNA was also purified by PCR column and a 1:2 dilution of the cDNA was used as a template for RACE PCR.

Fig 5.2 RNA on a formaldehyde Gel

Lane 1 purified dephosphorylated RNA

Lane 2 purified decapped RNA

28s and 18s rRNA were marked alongside the bands. The clear and sharp bands indicated that the RNA quality was still good.

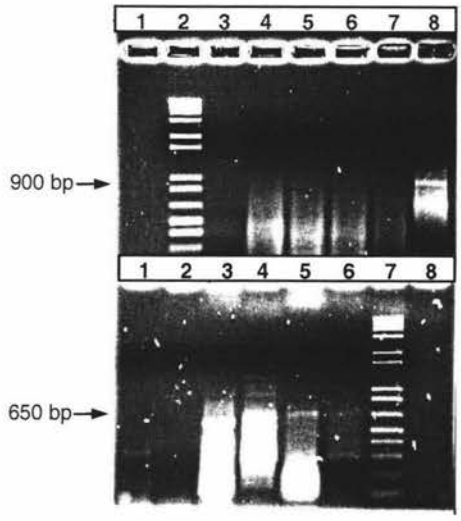


5.3 RACE OF *DOTA, DOTB, DOTC* AND *DOTD* GENES

RACE of *dotA*, *dotB*, *dotC* and *dotD* were carried out. HeLa total RNA was used a positive control of the entire RACE procedure; it should have a 900 bp product from the 5'RACE PCR and 1.8 kb from the 3' RACE PCR. The negative control was an aliquot of reaction mixture without template; it should not have any products from the RACE PCR. A touch down program was used for RACE PCR. The RACE PCR results of *dotA*, *dotB*, *dotC* and *dotD* are show in Fig 5.3.

In Fig 5.3, the 5'RACE positive control (top gel lane 8) and negative control (top gel lane 3) both gave expected results. There was a smear of products of 5' RACE PCR from *dotA*, *dotB*, *dotC* and *dotD* genes (top gel lanes 4-7). In contrast, there were some products (~ 650 bp) of 3' RACE PCR from *dotB*, *dotC* and *dotD* genes, but not from *dotA* (bottom gel lanes 2-5) and the positive control (bottom gel lane 1). However, there were also some faint products from 3'RACE the negative control. The products of the 3'RACE negative control were checked with nested PCR again as shown in Fig 5.4 bottom gel lane 5); there were no products from the nested PCR.

Fig 5.3 RACE of *dotA, dotB, dotC* and *dotD* genes



Top Gel: 5'RACE of dot genes

Lane 1 empty	Lane 5 *dotC*
Lane 2 1kb+ ladder	Lane 6 *dotB*
Lane 3 negative control	Lane 7 *dotA*
Lane 4 *dotD*	Lane 8 positive control

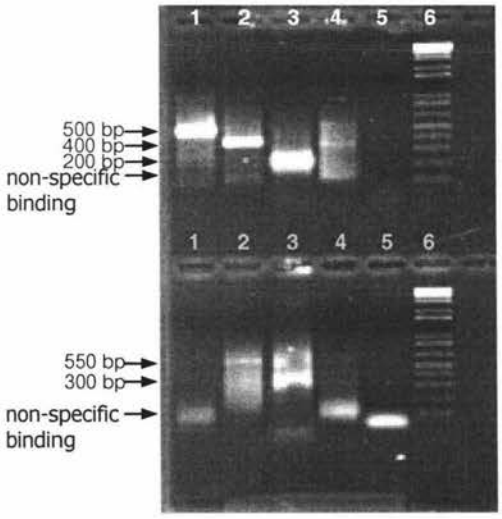
Bottom Gel: 3'RACE of dot genes

Lane 1 positive control	Lane 5 *dotD*
Lane 2 *dotA*	Lane 6 negative control
Lane 3 *dotB*	Lane 7 1kb+ ladder
Lane 4 *dotC*	Lane 8 empty

The RACE PCR products sizes are indicated along side the gel.

Nested RACE PCR was carried out to increase the specificity of the amplification. The nested RACE PCR was performed with the 5'and 3' GeneRacer^{TM} nested primers and gene specific primers and using the RACE PCR products as templates to get distinct RACE PCR products from these genes. The results are shown in Fig 5.4.

Fig 5.4 Nested RACE of *dotA, dotB, dotC and dotD*



Top gel: 5'nested PCR

Lane 1: *5'dotA*	Lane 4: *5'dotD*
Lane 2: *5'dotB*	Lane 5: negative control
Lane 3: *5'dotC*	Lane 6: 1kb+ ladder

Bottom gel: 3'nested PCR

Lane 1: *3'dotA*	Lane 4: *3'dotD*
Lane 2: *3'dotB*	Lane 5: negative control
Lane 3: *3'dotC*	Lane 6: 1kb+ ladder

The nested RACE PCR products sizes are indicated along side the gel.

There were clear PCR products from 5' nested RACE of *dotA*, *dotB* and *dotC* (Fig 5.4, top gel lanes 1-3). The 5' nested RACE of *dotD* had three different size bands (top gel lane 4). There was no product from the 5'nested PCR negative control (using 5'RACE PCR products as a template). For 3' nested RACE, *dotB* and *dotC* had PCR products

with high background (Fig 5.4, bottom gel lanes 2 and 3). Bands around 100 bp from *3'dotA*, *3'dotD* RACE (Fig 5.4, bottom gel lanes 1 and 4) and the 3' nested PCR negative control (3' RACE negative control as a template) were too small to be RACE products and were probably non-specific products or primer dimers. The *3'* RACE of *dotA* was repeated with a different gene specific primer after it was realized that the original one had three base pairs in the intron region at the 3'end of primer oligo. The *3'dotD* RACE was obtained with fresh RNA (Fig 5.5a).

Subsequently, the PCR products of 5' RACE of *dotA*, *dotB*, *dotC* and *dotD* were cleaned by PCR purification (Section 2.3.5) and the PCR products of 3'RACE of *dotA*, *dotB, dotC* and *dotD* were cleaned by gel extraction and purification (Section 2.3.7). These products were ligated with pGEM-T or pGEM-T Easy vector (Section 2.4.2) and transformed into *E. coli*. The transformants of the PCR products were screened by colony PCR with primers *T7* and *SP6* (Section 2.6.3).

The sequence results of the RACE PCR products of the four dot genes were analyzed by a BLASTn (www.ncbi.nlm.nih.govt/BLAST/) comparison with the published sequence (Genbank access number: gb|AF448056) (Bradshaw *et al.*, 2002). In general, the longest RACE sequence was used as a RACE result to identify the 5'or 3'ends of the UTR of the tested gene according to the manual, although in some cases, multiple transcriptional start or stop sites were evidenced (see in Section 5.4.2), they would have multiple PCR bands from the nested RACE. The 5' gene sequence end in the original sequence RACE PCR clones was identified by the 5' nested RACE primer oligo sequence tailed with GAAA (or complement oligo CTTT as shown in Fig 5.5). The 3'gene end was identified by poly dTs (18-24 dTs) (or complement oligo dAs) of the original sequence of the 3' nest RACE PCR clones. The RACE sequence alignments of *dotA* are shown below as an example (Figures 5.5 and 5.6), whilst those of *dotB, dotC* and *dotD* are shown in Appendix IV. The RACE results of *dotA, dotB, dotC* and *dotD* are summarized in Table 5.1

The sequence qualities of the RACE PCR products were good. Both 5' and 3'*dotA* RACE sequences have 99% identity to the published sequence. There are 2 A-G mismatches in 454 bp 5'RACE and 1 A-G mismatch in 427 bp 3'RACE. Two introns were covered in the *dotA* RACE sequences. The 5'UTR end of *dotA* is identified at 120

Fig 5.5 The 5' RACE of *dotA*

Top: RACE sequence; **Bottom**: Genbank sequence gb|AF448056. **The primers** using to amplify the RACE sequence are underlined. **The primer names** and **the 5'UTR end** (at the bottom end of the figure) are labelled in the text box.
ATG: translation start codon; Intron region; ⬚: mismatch

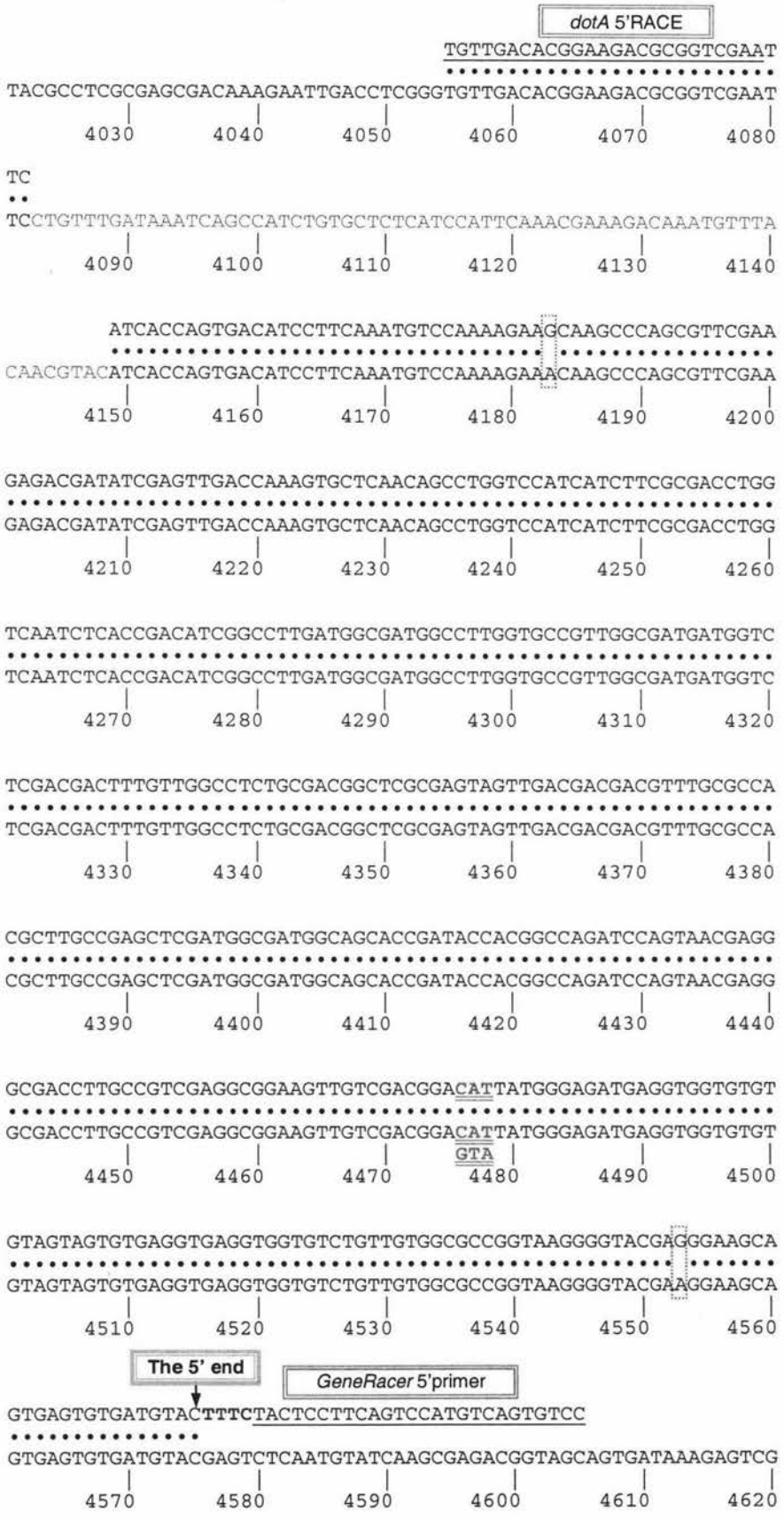
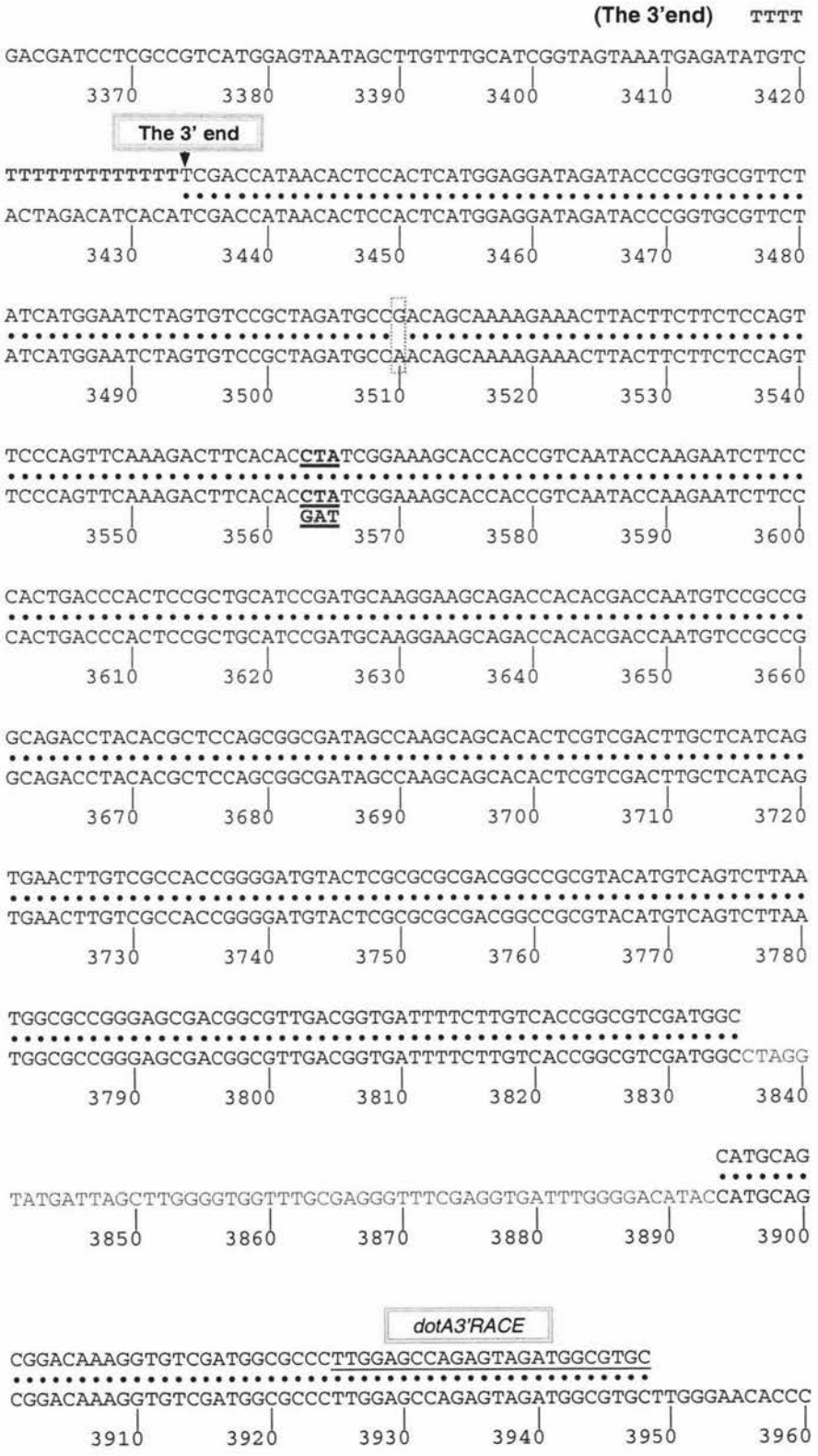


Fig 5.6 3'RACE of *dotA*

Top: RACE sequence; **Bottom**: Genbank sequence AF448056. **The primers** using to amplify the RACE sequence are underlined. **The primer names** and **the 3'UTR end** are labelled in the text box.
TAG: translation stop codon; Intron region; ⬚: mismatch.



bp upstream of the start codon and the 3' UTR end is identified at 107 bp downstream of the stop codon.

The 5' *dotB* RACE (326 bp) has 100% identity and the 3'*dotB* RACE has 98% identity to the published sequence. There are 2 gaps and one C-T and one A-G mismatches in the 521 bp 3' *dotB* RACE sequence. The 5'UTR end of *dotB* is identified at 40 bp upstream of the start codon and the 3' UTR end of *dotB* is identified at 182 bp downstream of the stop codon.

The *dotC* RACE sequence identities are the same as *dotB* RACE, 5'RACE (174 bp) 100% match and 3'RACE 98% match the published sequence. There are 4 gaps and one C-G mismatch in 269 bp 3' *dot C* sequence. The 5'UTR end of *dotC* is relatively long and it is identified at 273 bp upstream of the start codon and the 3' UTR end of *dotC* is identified at 129 bp downstream of the stop codon.

The 5'RACE of *dotD* has 99% identity to the published sequence. Two 3'RACE sequences were obtained from 3' *dotD* RACE. The sequence 3'dotD1 has 100% identity and another one 3'dotD2 has 99% identity to the published dothistromin gene sequences. There is one T-G mismatch in 417 bp 5' *dotD* RACE sequence and there are one A-G and two C-T mismatches in 3'dotD2 RACE sequence. The 5'UTR end of *dotD* is identified at 102 bp upstream of the start codon. There are two 3' UTR end sites identified by the sequences 3'dotD1 and 3' dotD2. The 3'dotD1 identifies the 3' UTR end site of *dotD* at 216 bp downstream of the stop codon and the 3'dotD2 sequence identifies the second 3'UTR end site of *dotD* at 257 bp downstream of the stop codon.

5.4 RACE AND cDNA OF THE FIVE NEWLY IDENTIFIED DOTHISTROMIN GENES

Compared to the *dotA, dotB, dotC* and *dotD* genes, these five new identified genes, *pksA, cypA, avfA, moxA* and *epoA* were not well characterized; *cypA* did not even have cDNA sequence. The gene specific primers for these genes were designed on the cDNA sequence (where known) or the putative exon region. For the *cypA* gene, RACE primers

Table 5.1 RACE results of *dot A, dotB, dotC* and *dotD* genes

Gene	Items	5'RACE	3'RACE
dotA	primers	GeneRacer™ 5'primer, GeneRacer™ 5' nested primer and *dotA5'RACE*	GeneRacer™ 3'primer, GeneRacer™ 3' nested primer and *dotA3' RACE*
	*Location in the genome sequence	**4575-4054 (intron 4082-4148)**	**3437-3948 (intron 3836-3893)**
	Size of the nested product	**454 bp**	**427 bp**
	UTR end	**- 120 bp**	**+ 107 bp**
dotB	primers	GeneRacer™ 5'primer, GeneRacer™ 5' nested primer and *dotB5'RACE*	GeneRacer™ 3'primer, GeneRacer™ 3' nested primer and *dotB3'RACE*
	*Location in the genome sequence	**7068-6743**	**8210-7690**
	Size of the nested product	**326 bp**	**521 bp**
	UTR end	**- 40 bp**	**+ 182 bp**
dotC	primers	GeneRacer™ 5'primer, GeneRacer™ 5' nested primer and *dotC5'RACE*	GeneRacer™ 3'primer, GeneRacer™ 3' nested primer and *dotC3'RACE*
	*Location in the genome sequence	**10551-10725**	**8888-8621**
	Size of the nested product	**174 bp**	**267 bp**
	UTR end	**-273 bp**	**+ 129 bp**
dotD	primers	GeneRacer™ 5'primer, GeneRacer™ 5' nested primer and *dotD5'RACE*	GeneRacer™ 3'primer, GeneRacer™ 3' nested primer and *dotD3'RACE*
	*Location of the genome sequence	**12001-12417**	**12743-13288** **12743-13329**
	Size of the nested product	**417 bp**	**545 bp** **587 bp**
	UTR end	**- 102 bp**	**+216 bp** **+257 bp**

*** Location of the sequence region with respect to the published sequence: GenBank** AF448056.

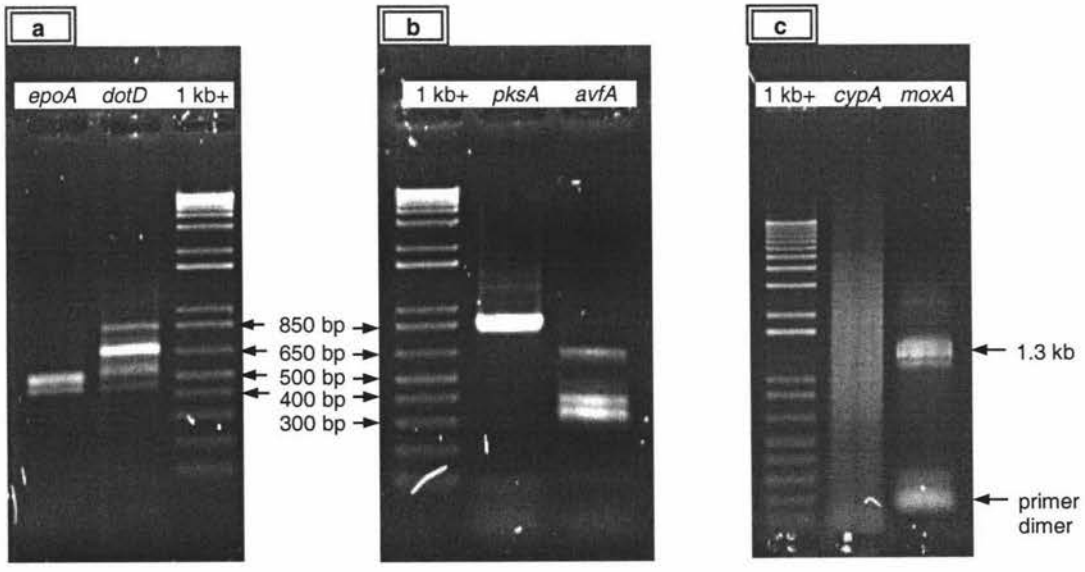
UTR ends are given upstream (-) of the start codon for 5'RACE or downstream (+) of the stop codon for 3'RACE.

were designed overlapping with each other, in order to get the complete cDNA sequence of the gene. Fresh RNA was extracted from *D. septosporum* strain NZE7 day 6 mycelia under conditions known to support dothistromin synthesis. The RACE procedure was repeated with fresh RNA and reverse transcribed with the GeneRacer™ Oligo dT primer.

5.4.1 The 3'RACE of the newly identified dothistromin genes

The 3'RACE was relatively easy compared to the 5'RACE. The nested PCR of 3'RACE of the newly identified genes (except 3'*cypA* RACE) worked straight away as shown in Fig 5.7. The PCR products from *avfA*, *epoA*, *pksA* and *moxA* were cloned and sequenced. The 3' RACE of *cypA* gene was subsequently obtained by one step RT-PCR with GeneRacer™ Oligo dT primer (RACE kit) and gene specific primer (*3'cypA*) and followed by nested PCR with 3'nested RACE primer and gene specific primer *3'cypA*. The nested PCR product was cloned and sequenced as the other PCR products. The sequences of the PCR products were aligned with the λKSA DNA sequence (which was submitted to Genbank recently, the access number is DQ149246) to identify the 3'end of the genes (by oligo dTs).

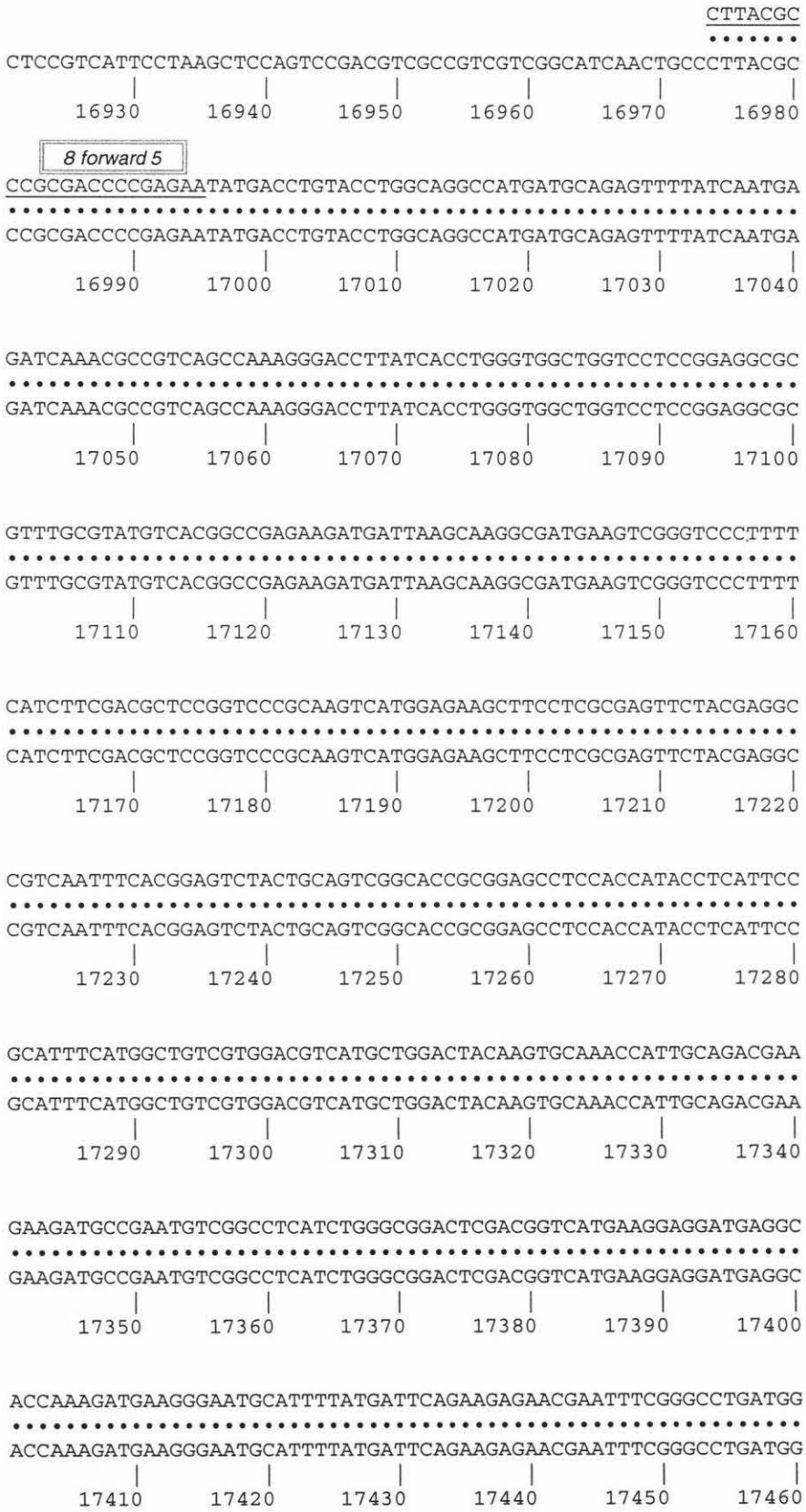
Fig 5.7 The 3'nested RACE PCR product of the new identified dothistromin genes

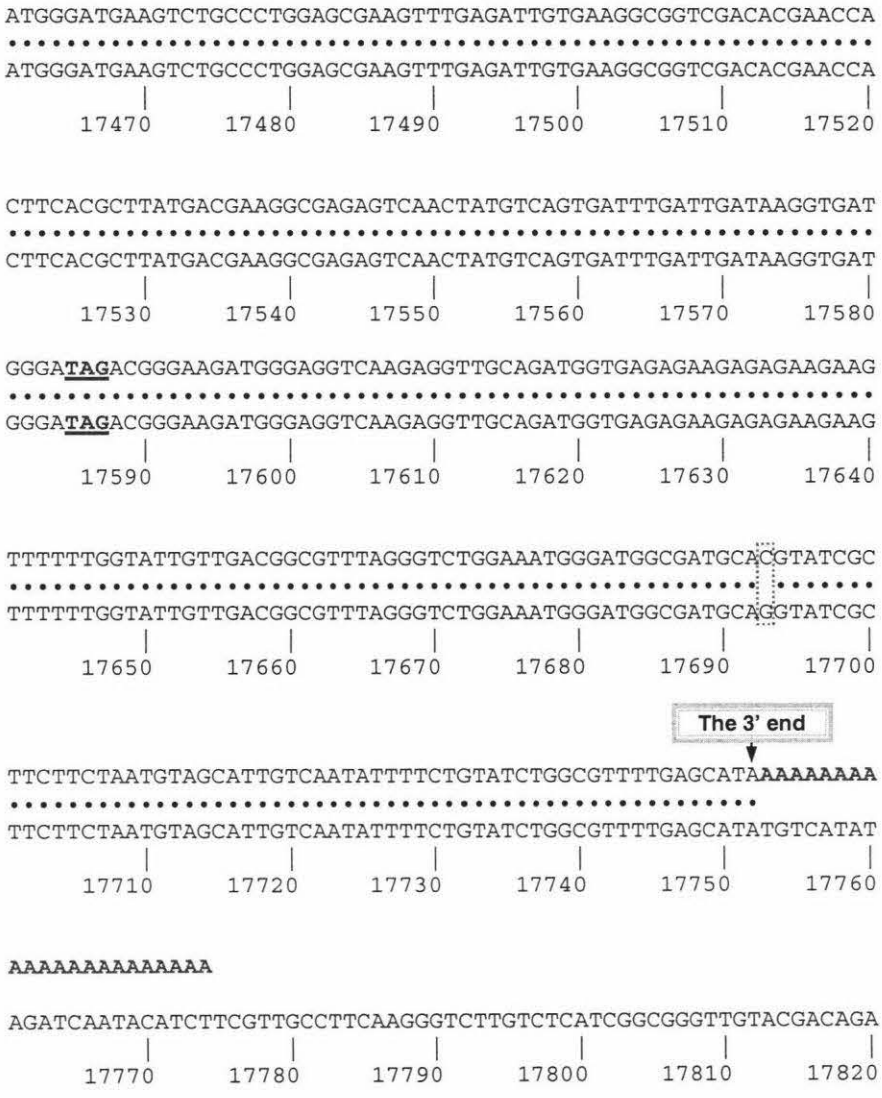


The 3'RACE sequence of *pksA* is shown in Fig 5.8 as an example, whereas those of *moxA, epoA, avfA* and *cypA* are shown in the Appendix IV. The 3'RACE results are summarized in Table 5.2 along with 5'RACE results.

Fig 5.8 The 3' RACE of *pksA*

Top sequence: the 3'RACE sequence of *pksA;* **Bottom sequence**: Genbank sequence DQ149246. **The primers** using to amplify the RACE sequence are underlined. **The primer names** and the **3'UTR end** are labelled in the text box.
⬚: mismatch. **TAG**: translation stop codon.




ATGGGATGAAGTCTGCCCTGGAGCGAAGTTTGAGATTGTGAAGGCGGTCGACACGAACCA
ATGGGATGAAGTCTGCCCTGGAGCGAAGTTTGAGATTGTGAAGGCGGTCGACACGAACCA
17470 17480 17490 17500 17510 17520
CTTCACGCTTATGACGAAGGCGAGAGTCAACTATGTCAGTGATTTGATTGATAAGGTGAT
CTTCACGCTTATGACGAAGGCGAGAGTCAACTATGTCAGTGATTTGATTGATAAGGTGAT
17530 17540 17550 17560 17570 17580
GGGATAGACGGGAAGATGGGAGGTCAAGAGGTTGCAGATGGTGAGAGAAGAGAGAAGAAG
GGGATAGACGGGAAGATGGGAGGTCAAGAGGTTGCAGATGGTGAGAGAAGAGAGAAGAAG
17590 17600 17610 17620 17630 17640
TTTTTTGGTATTGTTGACGGCGTTTAGGGTCTGGAAATGGGATGGCGATGCACGTATCGC
TTTTTTGGTATTGTTGACGGCGTTTAGGGTCTGGAAATGGGATGGCGATGCAGGTATCGC
17650 17660 17670 17680 17690 17700
The 3' end
TTCTTCTAATGTAGCATTGTCAATATTTTCTGTATCTGGCGTTTTGAGCATAAAAAAAAA
TTCTTCTAATGTAGCATTGTCAATATTTTCTGTATCTGGCGTTTTGAGCATATGTCATAT
17710 17720 17730 17740 17750 17760
AAAAAAAAAAAAA
AGATCAATACATCTTCGTTGCCTTCAAGGGTCTTGTCTCATCGGCGGGTTGTACGACAGA
17770 17780 17790 17800 17810 17820

The 3' RACE sequence of *moxA* is 1265 bp in length and confirms two putative introns, 54 bp and 58 bp respectively, in the 3' *moxA* region. There are two mismatches (one A-G and one C-T) compared to the gene DNA sequence. These two mismatches are located in the gene-coding region. The genomic DNA sequence of that region is more reliable, the mismatches of the RACE sequence may be caused by errors during PCR amplification. The 3' UTR end of *moxA* is identified at 141 bp downstream of the *moxA* stop codon (TAA).

The *epoA* 3'RACE sequence is 468 bp in length and covers an identified 51 bp intron. The sequence is 100% identical to the genomic DNA sequence. The 3'UTR end of *epoA* is identified at 155 bp downstream of the *epoA* stop codon. The *epoA* 3'RACE sequence is 70 bp overlapped with 3'RACE sequence of *avfA*.

The 3'RACE of *avfA* sequence quality was not good. There were 8 mismatches and gaps in 261 bp length. However, it still identified a 63 bp new intron, which covered the putative translation stop codon. The new predicted stop codon is 118 bp down stream of the original putative stop codon and the 3'UTR end of *avfA* is identified at 89 bp downstream of the new stop codon. The RACE result was checked by RT-PCR (Section 2.10.4.2) with primers *3'ordRACE* and *3'ord* (designed at the identified UTR end). The sequence of RT-PCR products matches completely the genomic DNA sequence (Appendix IV). Also, the 3'RACE result of *avfA* identifies 70 bp overlapping region with 3'RACE sequence of *epoA* on the identified genomic DNA sequence (Appendix IV).

The 3'RACE sequence of *cypA* is 810 bp in length and confirms a 53 bp predicted intron at 3' region of *cypA*. There is a G-A mismatch in the sequence compared to genomic DNA sequence. The 3'UTR end of *cypA* is identified at 149 bp downstream of the *cypA* stop codon.

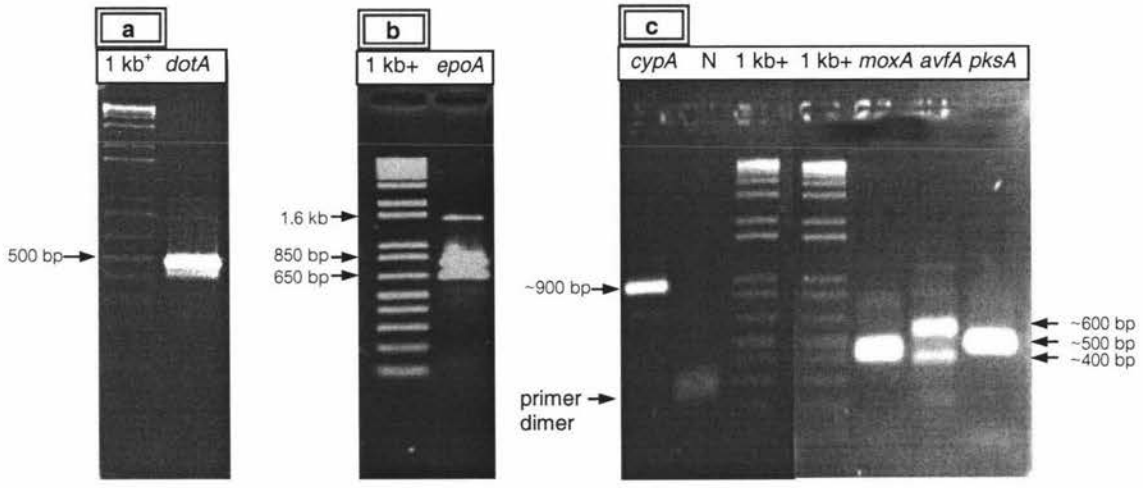
The 3' RACE of *pksA* is 778 bp in length. One C-G mismatch is identified in the sequence compare to genomic DNA sequence. The end of 3'UTR is identified 164 bp downstream of the *pksA* stop codon.

5.4.2 The 5'RACE of the newly identified dothistromin genes

The 5'RACE of the newly identified dothistromin genes was more difficult than 5'RACE of the four *dot* genes. Since *pksA* is a large gene (~ 8 kb), in order to get the completed 5'end of the genes, the RACE procedure was repeated with cDNA template obtained by reverse transcription with random primers (RACE kit, Invitrogen) instead of the oligo dTs. The 5' RACE of *dotA* was used as a positive control for the 5' RACE PCR of these five genes. The results are shown in Fig 5.9.

The 5'RACE of *dotA* had distinct products of the expected size even without nested RACE (compare Fig 5.9a to Fig 5.3). This result indicated that the new cDNA had a better quality. The 5'RACE PCR and nested RACE PCR were carried out on the five genes using this cDNA.

Fig 5.9 Nested PCR of 5' RACE of *epoA, moxA, avfA* and *pksA* gene



As shown in Fig 5.9 b and c, these five genes, *moxA*, *epoA*, *avfA*, *cypA* and *pksA*, all had clear products from the nested RACE PCR. The products were purified, cloned and sequenced as the other RACE PCR products. The sequences of the PCR products were all good quality.

There is a strong PCR product band (~ 450 bp) of the nested RACE of the *moxA* gene. The 5'RACE sequence of *moxA* is 419 bp in length and 100% identical to the known *moxA* DNA sequence. The end of 5'UTR is identified at 187 upstream of the *moxA* start codon.

The 5'nested RACE PCR of *epoA* had three different bands, which were separated and purified by gel purification (Section 2.3.7) followed by PCR to amplify enough DNA for cloning. The 1.6 kb band (Fig 5.9) turned out to be a polymer of the two small bands; when re-amplified, it gave two PCR bands as the two small PCR products in Fig 5.9b. Two small bands were cloned and sequenced. The sequence of the 650 bp band did not match the known *epoA* DNA sequence. The sequence of the 835 bp band matched the known *epoA* DNA sequence. This sequence is considered to be the 5'RACE result of *epoA*. There is one A-G mismatch in this sequence. The 5'UTR end of *epoA* is identified at 239 bp upstream of the *epoA* start codon.

There were two distinct PCR bands for the *avfA* gene. One band size was ~ 600 bp and another ~ 400 bp. Both bands were cloned and sequenced. Their sequences both matched the known *avfA* DNA sequence. There was no mismatch in the short sequence, which identified that the 5'UTR end of the *avfA* gene is at – 215 bp upstream of the start codon. There were one A-T mismatch and a 6 bp gap in the longer sequence, which identified that the 5'UTR end of the *avfA* gene is at –353 bp upstream of the start codon. Whether the *avfA* gene has one or two 5'UTR ends requires further confirmation (see discussion).

There is one clear band (~900 bp) of the 5' nested RACE PCR of the *cypA* gene. The sequence of the PCR product is 990 bp in length. There is one bp gap in the sequence compared to the known *cypA* DNA sequence. One 51 bp new intron was identified and the putative start codon was covered in the new intron region. The new predicted start codon is 200 bp upstream of the original putative start codon and the 5'end UTR of *cypA* is at 100 bp upstream of the new start codon.

The 5' nested RACE of *pksA* also had a strong PCR product band (~ 500 bp). The sequence of the PCR product was 470 bp in length and 100% identical to the known *pksA* DNA sequence. The RACE sequence covers a 49 bp intron region. The 5' UTR end of the *pksA* gene was identified at 239 bp upstream of the *pksA* start codon. The 5' RACE sequence of *pksA* is shown in Fig 5.10 as an example.

The RACE results of these five dothistromin genes are summarized in Table 5.2.

Fig 5.10 The 5' RACE of *pksA*

Top: 5'RACE sequence of *pksA*; **Bottom**: Genbank sequence DQ149246. **The primers** using to amplify the RACE sequence are underlined. **The primer names** and **the 5'UTR end** are labelled in the text box.
ATG: translation start codon; Intron region

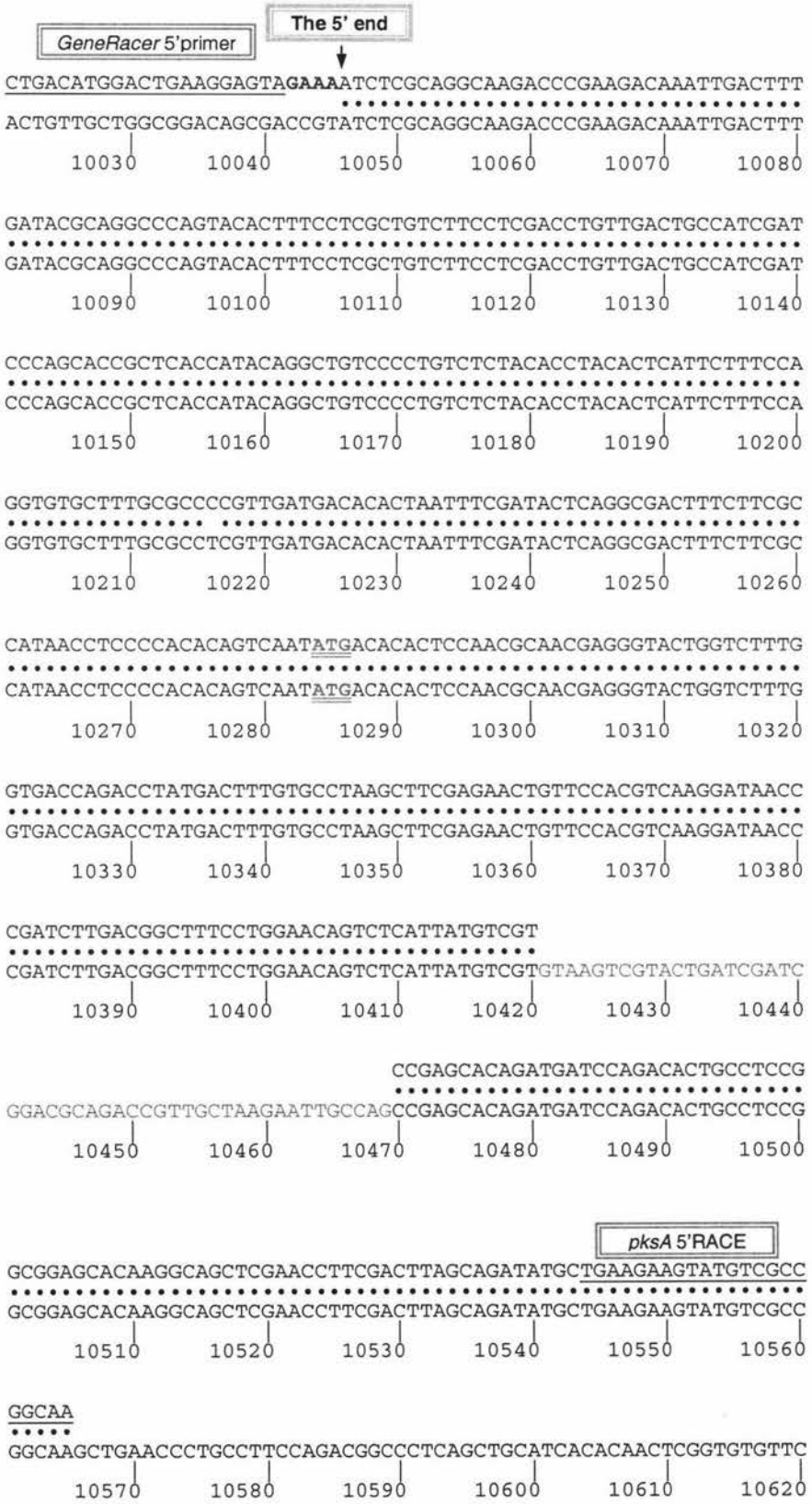


Table 5.2 RACE results of *moxA, epoA, avfA, cypA* and *pksA* genes

Gene	Items	5'RACE	3'RACE
moxA	primers	GeneRacer™ 5'primer, GeneRacer™ 5' nested primer and *moxA5'RACE*	GeneRacer™ 3'primer, GeneRacer™ 3' nested primer and *moxA3'RACE*
	*Location in the genome sequence	**3817-4236**	**1746-3073** Two introns: 1983-2038 2265-2313
	Size of the nested product	**419 bp**	**1265 bp**
	UTR end	**-187 bp**	**+141 bp**
epoA	primers	GeneRacer™ 5'primer, GeneRacer™ 5' nested primer and *5'epoARACE*	GeneRacer™ 3'primer, GeneRacer™ 3' nested primer and *epoA3'RACE*
	*Location in the genome sequence	**4340-5175**	**5528-6049** intron: 5683-5735
	Size of the nested product	**835 bp**	**468 bp**
	UTR end	**- 239 bp**	**+ 155 bp**
avfA	primers	GeneRacer™ 5'primer, GeneRacer™ 5' nested primer and *avfA5'RACE*	GeneRacer™ 3'primer, GeneRacer™ 3' nested primer and *avfA3' RACE*
	*Location in the genome sequence	**6742-7382** **6744-7244**	**5979-6303** #intron: 6129-6192
	Size of the nested product	**502 bp** **640 bp**	**261 bp**
	UTR end	**-215 bp** **- 342 bp**	**+ 89 bp**
cypA	primers	GeneRacer™ 5'primer, GeneRacer™ 5' nested primer and *cypA5'RACE*	GeneRacer™ 3'primer, GeneRacer™ 3' nested primer and *cypA3'RACE*
	*Location in the genome sequence	**8683-9723** #intron: 9465-9515	**7834-8696** intron: 8255-8307
	Size of the nested product	**990 bp**	**810 bp**
	UTR end	**- 100 bp**	**+ 149 bp**
pksA	primers	GeneRacer™ 5'primer, GeneRacer™ 5' nested primer and *5'pksARACE*	GeneRacer™ 3'primer, GeneRacer™ 3' nested primer and *pksA3'RACE*
	*Location in the genome sequence	**10047-10565** intron: 10421-10469	**16974-17752**
	Size of the nested product	**470 bp**	**778 bp**
	UTR end	**- 239 bp**	**+ 164 bp**

* Location of the sequence region with respect to the genomic sequence Genbank: DQ149246.
#: new identified intron or confirmed intron.
UTR ends are given upstream (-) of the start codon for 5'RACE or downstream (+) of the stop codon for 3'RACE.

5.5 THE REGULATORY MOTIFS OF THE IDENTIFIED DOTHISTROMIN GENES

The putative binding motifs of several fungal transcription factors, BrlA (5'-MRAGGGR-3'), AbaA (5'-CATTCY-3'), PacC (5'-GCCARG), AreA (5'-HGATAR-3') and AflR (5'-TCGN$_5$(or N$_{11}$)CGR) found in AF genes (Ehrlich *et al.*, 2002), were analysed by Vector NTI to identify if they exist in the untranscribed gene regions of the dothistromin genes (coding strand, Appendix V). BrlA and AbaA regulate expression of structural genes involved in conidiation and other developmental process (Ehrlich *et al.*, 2002). PacC is a global transcriptional regulator in response to pH (Calvo *et al.*, 2002). AreA is a repressor of nitrate–mediated gene transcription, for example in AF biosynthesis (Chang *et al.*, 2000). AflR is a major regulator of AF biosynthesis (Woloshuk *et al.*, 1994).

The *dotA* and *dotB* genes share a 2165 bp untranscribed intergenic region; *dotC* and *dotD* share an 1276 bp untranscribed intergenic region; *moxA* and *epoA* share an 104 bp untranscribed intergenic region; *cypA* and *pksA* share a 324 bp untranscribed intergenic region. There is a 552 bp untranscribed region between 5' UTR end of *avfA* and 3'UTR end of *cypA*.

In the nine dothistromin genes, two of them (*dotB* and *dotD*) contain a BrlA binding motif, five of them (*dotB*, *dotC, dotD, avfA* and *cypA*) contain an AbaA binding motif, six of them (*dotA*, *dotB*, *dotC*, *dotD*, *avfA* and *cypA*) contain a PacC binding motif and five of them (*dotA*, *dotB*, *dotC, avfA* and *cypA*) contain an AreA binding motif, eight of them (except *avfA*) contain an AflR binding motif and six of them (*dotA, dotB, dotC, dotD, pksA* and *cypA*) contain an expanded AflR-like binding motif (5'-TCGN$_{11}$CGR) in the 5'untranscribed regions. The results are summarized in Table 5.3 and the sequences of the 5'untranscribed regions and the binding motifs are included in the Appendix V.

Table 5.3 the putative regulatory motif of the dothistromin genes.

Genes	BrlA (MRAGGGR)	AbaA (CATTCY)	PacC (GCCARG)	AreA (HGATAR)	AflR ($TCGN_5CGR$)	AflR-like ($TCGN_{11}CGR$)
dotA	-	-	++	++	++++++	+++
dotB	+	++	++	+++++	+++++	+++
dotC	-	+++	+	+	++	+++++
dotD	+	+	+++	-	+	+++
pksA	-	-	-	-	++	++
cypA	-	+	+	+	++	+
avfA	-	+	+	+	-	++
epoA	-	-	-	-	++	-
moxA	-	-	-	-	++	-

+: indicates the presence and number of the binding motifs of each gene.

-: indicates there is no binding motif in that gene untranscribed region

5.6 DISCUSSION

The RACE kit (Invitrogen) provided a way to ensure that the full-length (not partial) mRNA of genes was transcribed and identify the untranslated regions of genes. It has been widely used in the characterization of newly identified genes, such as *pksL1* (Feng and Leonard, 1995), *CTB1* (Choquer *et al.*, 2005), *pks1* (Zhang *et al.*, 2003) and *chiB* (Jaques *et al.*, 2003) genes.

5.6.1 RNA purification with PCR column

The RNA integrity is essential to obtain a completed cDNA sequence. During the RACE procedure, a good positive control is necessary to check if every reaction of the RACE works. In contrast, a negative control is useful but not as important as positive control, because normally RACE PCR products are really hard to get.

The PCR purification kit was successfully used in RNA and single strand cDNA purification; it provided a quick and easy way to manipulate RNA compared to the method recommended by the manufacturer. However, the disadvantage of the DNA purification kit is that 30 µl DEPC-H_2O need to be used to elute RNA after each purification. The purified RNA was diluted after purification. More total RNA should

be used at the beginning, in this project 7 μg total RNA (Section 2.9) was used instead of 5 μg recommended by the manual. As mentioned above, the cDNA was diluted but it still works well for RACE PCR.

5.6.2 RACE PCR

The RACE results were obtained through PCR amplification. In order to overcome the disadvantage of general PCR, such as non-specific binding and incorrect sequences, the recommendations of the RACE manual were followed. First of all, RACE requires designing high annealing temperature (>72°C) primers of each targeted gene. For 5'RACE of *pksA* and *epoA*, new designed primers had clear PCR products. High Fidelity *Taq* polymerase was used in the RACE PCR to help ensure fewer mismatches in the sequence. Also the special touch down program was used to amplify RACE products with higher specificity of primer binding. If there were not enough PCR products, a nested PCR of the RACE products was carried out at the high annealing temperature (65°C) to obtain enough PCR products for cloning. All these strategies help to ensure that the RACE PCR products are specific for the targeted gene mRNA.

5.6.3 RACE of the dothistromin genes

The PCR products were cloned and sequenced. In order to get the correct end of the gene, at least 10 clones of different size products were sequenced. The longest sequence that matched the genomic DNA sequence was used as the RACE result. Although High Fidelity *Taq* polymerase was used in RACE PCR to avoid mismatches, there are still some mismatches in some RACE sequences. The mismatches may be caused during the nested PCR with *Taq* polymerase. However the aim of the RACE sequence was to identify the 5' and 3' UTR ends, so one or two mismatches in the sequence will not change the results. Some RACE sequences cover (5'RACE of *dotA*, *pksA*) or identify (3'RACE of *avfA* and 5'RACE of *cypA*) intron regions, which confirm that the RACE sequence is obtained from mRNA.

Some genes may have multiple transcription start or stop sites, such as 5'*avfA* and 3'*dotD*. In the 3' RACE results, the *dotD* gene had two ends sites at the 3'end according to the RACE PCR products sequence. Normally, the gene 3'end is marked by the oligo

dT sequence, both RACE sequences included oligo dT at the end followed by the GeneRacer 3'primer sequence which suggested that the sequences were obtained from the 3'end of the mRNA of *dotD*. In the 5'RACE results, *avfA* had two end sites according to the RACE sequences. The sizes of the RACE products are matched the difference between the bands on the gel. For the longer sequence of 5'RACE of *avfA*, there is a 6 bp gap at the 5'end and a mismatch (A-T) 5'bp upstream of the gap. But the other 5' RACE sequences of *avfA*, that covered this region, exactly matched the gene DNA sequence. The gap may have been caused by PCR procedure. The longest sequence of 5'*avfA* RACE was confirmed by RT-PCR (result did not show). However, these results may be better confirmed with another method, such as northern hybridization.

Two genes' (*cypA* and *avfA*) previous predicted start or stop codon were changed according to RACE results and new introns were identified at the 5'end of *cypA* and 3'end of *avfA*. Two genes' (*avfA* and *epoA*) 3'UTR were overlapped 70 bp on the genomic DNA sequence. The results were confirmed by sequencing the RT-PCR products with the primers designed at the 3'end of these two genes and the 3'gene race primers. The 3'end overlapping of two genes is unusual in fungal gene clusters.

5.6.4 The regulatory motif of identified dothistromin genes

Five putative transcription regulators were analysed in the untranscribed region. A putative AflR binding motif was present upstream of all nine identified dothistromin genes. This suggests that in dothistromin biosynthesis, there may be an AflR-like transcription regulator. AflR is a key regulator of aflatoxin biosynthesis and its *cis*-acting sites are required for transcriptional activation of four aflatoxin genes *stcU, avnA, pksA* and *nor-1* (Miller *et al.*, 2005). The consensus AflR *cis*-acting sites in the AF cluster were also studied and it was shown that not all the AflR binding sites are functional; of three AflR binding sites upstream of the *pksA* gene, only two of them were required for *pksA* activity (Ehrlich *et al.*, 2002). Some dothistromin genes contain several consensus AflR binding sites, such as *dotA*, *dotB* and *dotC*, which contain at least five AflR binding sites.

The putative PacC binding motifs were identified in six genes. The other three genes (*epoA*, *moxA* and *pksA*) also contained a PacC binding motif, but located in 5'UTR region. It is not known if any of these are functional. It is not certain if PacC is involved in dothistromin biosynthesis. However, the *cis*–acting PacC site upstream of *pksA* gene in AF biosynthesis had a positive effect on *pksA* expression at acid pH and a negative effect at basic pH (Ehrlich *et al.*, 2002).

There are five dothistromin genes that contain putative AreA binding motifs. AreA is a regulator involved in nitrogen metabolism (Calvo *et al.*, 2002). The *areA* gene has been cloned and well characterized in *A. parasiticus.* AreA bound to the *aflR-aflJ* intergenic region of aflatoxin biosynthesis in *A. parasiticus*. It was assumed that AreA might regulate the expression of *aflR* or *aflJ* (Chang *et al.*, 2000). The presence of a putative AreA binding motif in five dothistromin genes suggests it may also participate in regulation of dothistromin biosynthesis in *D. septosporum.*

BrlA and AbaA are transcriptional regulators that govern expression of structural genes involved in conidiation and other developmental processes (Calvo *et al.*, 2002; Ehrlich *et al.*, 2002). BrlA was found regulating both *pksA* in AF biosyntheis and *wA* (*pks*) gene in *A. nidulans* (Ehrlich *et al.*, 2002). However, the BrlA binding site is only identified in *dotB* and *dotD* untranscribed regions, not in the other dothistromin genes, including *pksA.* A putative AbaA binding site was identified in five dothistromin genes, but not in *epoA*, *moxA* and *pksA*. There is no report about the role of AbaA being involved in AF/ST biosynthesis.

The dothistromin gene cluster is a new cluster compared to the AF/ST cluster. Although three genes (*dotA*, *pksA* and *epoA*) were characterized by gene disruption, only two of them (*dotA* and *pksA*) were confirmed to be involved in dothistromin biosynthesis. The RACE results shed light on the regulation of dothistromin biosynthesis. However, these results are still not enough to understand the dothistromin gene cluster. The presence of an AflR binding site upstream of each of the identified dothistromin genes implies that there may be an *aflR* homolog in the dothistromin gene cluster that plays the same role as *aflR* in AF/ST biosynthesis. The binding sites need to be characterized individually as was done in AF biosynthesis.

CHAPTER SIX: CONCLUSIONS AND FUTURE WORK

6.1 CONCLUSION AND FUTURE WORK

The aims of this project were to elucidate *pksA* and *epoA* gene function and to further characterize genes involved in dothistromin biosynthesis by RACE analysis.

Both *pksA* and *epoA* are located in the λKSA clone and they were each determined to be single copy genes in *D. septosporum* by Southern hybridization. The *pksA* gene was disrupted by replacing two conserved domains KS and AT with *hph* gene cassette. The *pksA* mutants lost the ability to produce dothistromin. The sporulation of *pksA* mutants was also reduced dramatically. In *A. nidulans,* the relationship between sporulation and ST production was analysed using mutants blocked at different stages of biosynthesis. The earlier the biosynthetic step that the blocked gene was involved in, the lower the sporulation of the mutant (Wilkinson *et al.*, 2004). Currently, two genes *pksA* and *dotA* are confirmed to be dothistromin biosynthetic genes and they are involved in a very early stage and a middle stage. Careful assessment of the *dotA* mutants' sporulation rate would provide evidence if the dothistromin production is related to sporulation in *D. septosporum.*

PksA has six conserved domains (KS-ST-ACP-ACP-ACP-TE). The presence of three ACP domains is not usual in fungal PKS proteins. What their function is, and whether they are redundant in function (as are the two ACP domains in *A. nidulans* WA PKS, (Fujii *et al.*, 2001)) needs further analysis. In the previously identified dothistromin genes, *dotD* is a homolog of the PksA TE domain whilst PksA in *D. septosporum* also contained a TE domain of its own. The *pksA* mutants confirmed that the *pksA* gene participates in a very early step prior to NOR formation. Therefore, it would be of interest to find out if *dotD* has any role in dothistromin biosynthesis.

The other uncharacterized dothistromin genes (*moxA*, *cypA* and *avfA*) located in the λKSA clone all have corresponding homologs in the AF/ST cluster whose functions in AF/ST biosynthesis have been characterized by gene disruption. The *avfA* homolog (*aflI*) in *A. parasiticus* is required for conversion of AVR to VHA (Yu *et al.*, 2000); the homologs of *moxA* (*moxY* = *aflw*) and *cypA* (*cypX* = *aflv*) in *A. parasiticus* participate in converting AVF to HVN (1-hydrooxyversicolorone) (Wen *et al.*, 2005). It is predicted that dothistromin genes have the same functions as their homologs in AF/ST

biosynthesis based on the *pksA* and *dotA* gene disruption results. If this hypothesis is right, further characterization of these three genes by gene disruption will provide more evidence that dothistromin biosynthesis is homologous to AF biosynthesis.

Disruption of the *epoA* gene could not provide any clues about its function and could not confirm whether it is a dothistromin biosynthetic gene. However, the position in which it is located on the λKSA clone suggests that it could be. Located between *moxA* and *avfA*, it is only 104 bp away from *moxA* at the 5' UTR end and the 3'UTR end was 70 bp overlapped with that of *avfA*. The similarity of gene expression to the two confirmed dothistromin genes (*pksA* and *dotA*) also implies that it is a dothistromin biosynthetic gene. Further characterization of the *epoA* mutants will be very helpful to understand the function (if any) of EpoA in dothistromin biosynthesis. A method such as HPLC or NMR could be used that can distinguish slight differences in the molecular structure between dothistromin and its precursors, for the further characterization of the *epoA* mutants.

If the *epoA* gene turns out to be dothistromin biosynthetic gene and is involved in a very late step in dothistromin biosynthesis, there will be three gene-disrupted mutants representing three different stages in dothistromin biosynthesis: *pksA*-early stage; *dotA*-middle stage and *epoA*-late stage. These results will be very helpful to draw a big picture on dothistromin biosynthetic steps. The steps after the *dotA* gene are particularly interesting as this is where the dothistromin biosynthetic steps differ from those of AF/ST biosynthesis. The *pksA* and *dotA* disruption results and the homologous genes of the AF/ST cluster identified in *D. septosporum* imply that before the *dotA* step, the dothistromin biosynthesis is highly similar to AF/AT biosynthesis, but after that it is not known, although Henry and Townsend have proposed a pathway (Henry and Townsend, 2005). So if the *epoA* gene is involved in dothistomin biosynthesis, it may be very helpful for understanding the late stages.

The *dotC* gene was proposed to encode a MFS transporter, which only has a homolog (*aflT*) in the AF cluster but not the ST cluster (Bradshaw *et al.*, 2002). The gene disruption of *dotC* is currently underway (Feng, personal communication) to characterize the function of *dotC* in dothistromin biosynthesis. The *dotB* gene has a homolog (*stcC*) in the ST cluster but not the AF cluster. However, due to lack of

information on the role of *stcC*, *dotB* is a mystery compared to the other identified genes. The *dotB* gene product was proposed to function before the *dotA* gene (Bradshaw *et al.*, 2002). What its function really is needs to be characterized by gene disruption.

The putative transcriptional regulatory protein binding sites in the identified dothistromin genes imply that there may be an *aflR*-like gene that exists in dothistromin biosynthesis. However, so far only two genomic DNA λ clones of *D. septosporum* have been identified and sequenced. The two λ clones contained a total of 31 kb genome sequence but did not contain an *aflR*-like gene. The dothistromin gene cluster was proposed to be 60-70 kb in length based on the size of the AF/ST gene cluster. It is unknown how far apart the two identified λ clones are and where they are located in the dothistromin gene cluster. However, separation of *D. septosporum* chromosomes on a CHEF gel showed that both *pksA* and *dotA* are located on a 1.3 Mb mini-chromosome (Bradshaw *et al.*, unpublished). The *pksA* and *dotA* genes are single copy genes in *D. septosporum* and they are located on different λ clones. The CHEF results therefore indicate that the dothistromin gene cluster is located on the 1.3 Mb mini-chromosome (Bradshaw *et al.*, unpublished). Screening of a *D. septosporum* cosmid library is currently underway (Zhang, personal communication) to identify more dothistromin genes. Hopefully, an *aflR*-like gene could be identified in the dothistromin cluster. This will be very helpful to understand regulation in dothistromin biosynthesis. The identification of further dothistromin cluster genes will provide a clue about which of the genes are linked and how far between the two sequenced λ clones. If the completed dothistromin gene cluster could be identified and that will make the dothistromin biosynthesis much more clear.

In an evolutionary view, the highly similarity between the dothistromin gene cluster and the AF/ST gene cluster raises interesting questions. *D. septosporum* is not evolutionarily closely related to *A. nidulans* and *A. parasticus* as it is in a different ascomycete class. However, the dothistromin cluster is the most homologous cluster to the AF/ST gene cluster known and their biosynthetic steps are very similar to each other based on the current knowledge and analysis of toxin-deficient mutants. Both dothistromin and afaltoxin are considered to be secondary metabolite toxins. Dothistromin is thought to be the cause of *Dothistroma* needle blight, but no functional role is known for AF/ST, and they are produced in saprophytic (not pathogenic) fungi. Continued research on the

dothistromin gene cluster may lead to knowledge of how such similar toxins envolved to have such different biological roles.

Research on dothistromin gene cluster may also provide a way to control the forest disease *Dothistroma* needle blight. The dothistromin deficient mutants (*pksA* mutants) will eventually allow identification of the precise role of dothistromin in the pathogenicity of *D. septosporum* to *P. radiata* possibly underpinning a new approach to control of this disease in pine forests.

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Appendix I MEDIA

All media were prepared with Milli-Q water and sterilized by autoclaving at 121 °C for 15 minutes. Media were cooled to approximately 50°C before addition of antibiotics.

A1.1 *E. coli* Media

Luria Broth (LB) (g/L): Tryptone (Becton, Dickison and company), 10; NaCl, 5; Yeast extract (Becton, Dickison and company), 5

LB solid media (g/L): Tryptone, 10; NaCl, 5; Yeast extract, 5; Agar, 15.

Selective LB media

Supplements were added at the following final concentrations.

(µg/mL): Ampicillin, 100; Kanamycin sulphate, 50; isopropylthio-β-D-galactoside (IPTG), 30; 5-bromo 4-chloro 3-indolyl-β-D-galactoside (X-gal), 60

A1.2 *D. septosporum* Media

***D. septosporum* media** (DM)

(g/L): Malt extract (Oxoid), 50.0; Nutrient agar (Oxoid), 28.0.

Low *D. septosporum* Broth (Low DB)

(g/L) Nutrient broth (Oxoid), 23; Malt extract (Oxoid), 23.

***D. septosporum* Sporulation Media** (DSM)

(g/L) Malt extract (Oxoid), 15; Yeast extract (BD), 5; Agar, 20.

A1.3 *D. septosporum* Transformation Media

***D. septosporum* Top media** (DM Top)

(g/L): Malt extract, 50.0; Nutrient Agar, 11.2: Sucrose, 273.9 (0.8 M).

Osmotically Stabilised DM (DM Suc)

(g/l): Malt extract, 50.0; Nutrient Agar, 28.0: Sucrose, 273.9 (0.8 M).

Selective DSM and DM

Media used to select for hygromycin resistant *D. septosporum* transformants contained (µg/ml): Hygromycin B (Sigma), 70.

Appendix II COMMON BUFFERS AND SOLUTIONS

All solutions were prepared with Milli-Q water and sterilized by autoclaving at 121°C for 15 minutes, unless otherwise stated.

A2.1 Common Buffers and solutions

TE Buffer

10 mM Tris-HCl and 1mM Na_2EDTA (TE 10:1) prepared from 1 M Tris-HCl (pH 8.0) and 250 mM Na_2EDTA (pH 8.0) stock solutions

1 x TBE Buffer

89 mM Tris-HCl, 2.5 mM Na_2EDTA and 89 mM Boric acid (pH 8.3).

Ethidium Bromide

Agarose gels were stained in ethidium bromide prepared as follows: 1 µl of 10 mg/ml stock per 10 ml of Milli-Q water to give a final concentration of 1 µg/ml.

RNAseA (DNAse free)

10 mg/mL RNAse was dissolved in 0.01M Sodium acetate (pH 5.2) and placed in a boiling water bath for 15 minutes. This was cooled slowly to room temperature and 0.1 volumes of Tris-HCl (pH 7.4) added and stored at -20°C.

Gel Loading Buffer (10x)

2 M Urea, 50% (v/v) glycerol, 50 mM Tris acetate, 0.4% (w/v) bromophenol blue and 0.4% (w/v) xylene cyanol.

1×TNE buffer

10 mM Tris-HCl, 1 mM Na_2EDTA and 100 mM NaCl, pH 7.4

A2.2 Genomic DNA isolation Buffers

Lysis buffer

40 mM Tris-Acetate pH 7.8; 20 mM Sodium Acetate; 1 mM EDTA; 1% SDS

CTAB buffer

2% CTAB (w/v), 1% PVP40 (w/v), 5 M NaCl, 0.5 M EDTA, 1 M Tris-HCl

A2.3 Reagents for *D. septosporum* Transformation

OM buffer: 1.4 M $MgSO_4 \cdot 7H_20$, with 10 mM Na_2HPO_4/100 mM NaH_2PO_4 buffer, pH 5.8

ST buffer: 1.0 M Sorbitol, 100 mM Tris-HCl (pH 8.0).

STC Buffer: 1.0 M Sorbitol, 50 mM Tris-HCl (pH 8.0), 50 mM $CaCl_2$.

40% PEG: 40 g PEG 6000 in 100 ml STC buffer

Glucanex: 10 mg/ml in OM buffer

A2.4 Reagents for Southern Blotting and Hybridization

Denaturing Solution: 500 mM NaOH, 500 mM NaCl

Neutralising Solution: 500 mM Tris (pH 7.4), 2 M NaCl

20 x SSC: 3 M NaCl, 0.2 M tri-sodium citrate, pH 7.0

Wash Solution I: 2 x SSC, 0.1% SDS

Wash Solution II: 0.5 x SSC, 0.1% SDS

Maleic Acid buffer: 100 mM Maleic Acid, 150 mM NaCl, pH 7.5

Washing Buffer: 100 mM Maleic Acid, 150 mM NaCl, pH 7.5; 0.3 %(v/v) Tween-20

Blocking solution: 1% blocking reagent (Roche Applied Science), in Maleic acid buffer

Antibody solution: Anti-Digoxigenin AP diluted 1:10000 in blocking solution

Detection buffer: 100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5

Stripping buffer: 0.2 M NaOH, 0.1% (v/v) SDS

2 x SSC: 0.3 M NaCl, 0.02 M tri-sodium citrate pH 7.0

A2.5 Reagents for Northern Blotting and Hybridization

DEPC-H_2O: 0.01% DEPC in MilliQ H_2O, sterilise twice.

10 xMOPS buffer: 200 mM Na-MOPS, 50mM NaOAc, 10 mM EDTA

BPB Loading dye: 0.2% bromophenol blue dissolved in 1 x MOPS buffer.

20 x SSC: 3 M NaCl, 0.2 M tri-sodium citrate in 0.01% DEPC-treated H_2O, pH 7.0.

100x Denhardts buffer: 1% Ficoll, 1% BSA, 1% PVP40

Hybridisation Solution (made fresh)

6 x SSC, 2 x Denhardts buffer, 0.1% SDS, 0.1% pre-boiled salmon sperm DNA (Sigma).

Wash Solution I: 1 x SSC, 0.1% SDS

Wash Solution II: 0.2 x SSC, 0.1% SDS

Appendix III PARTIAL SEQUENCE OF THE *EPOA* GENE DISRUPTION VECTOR

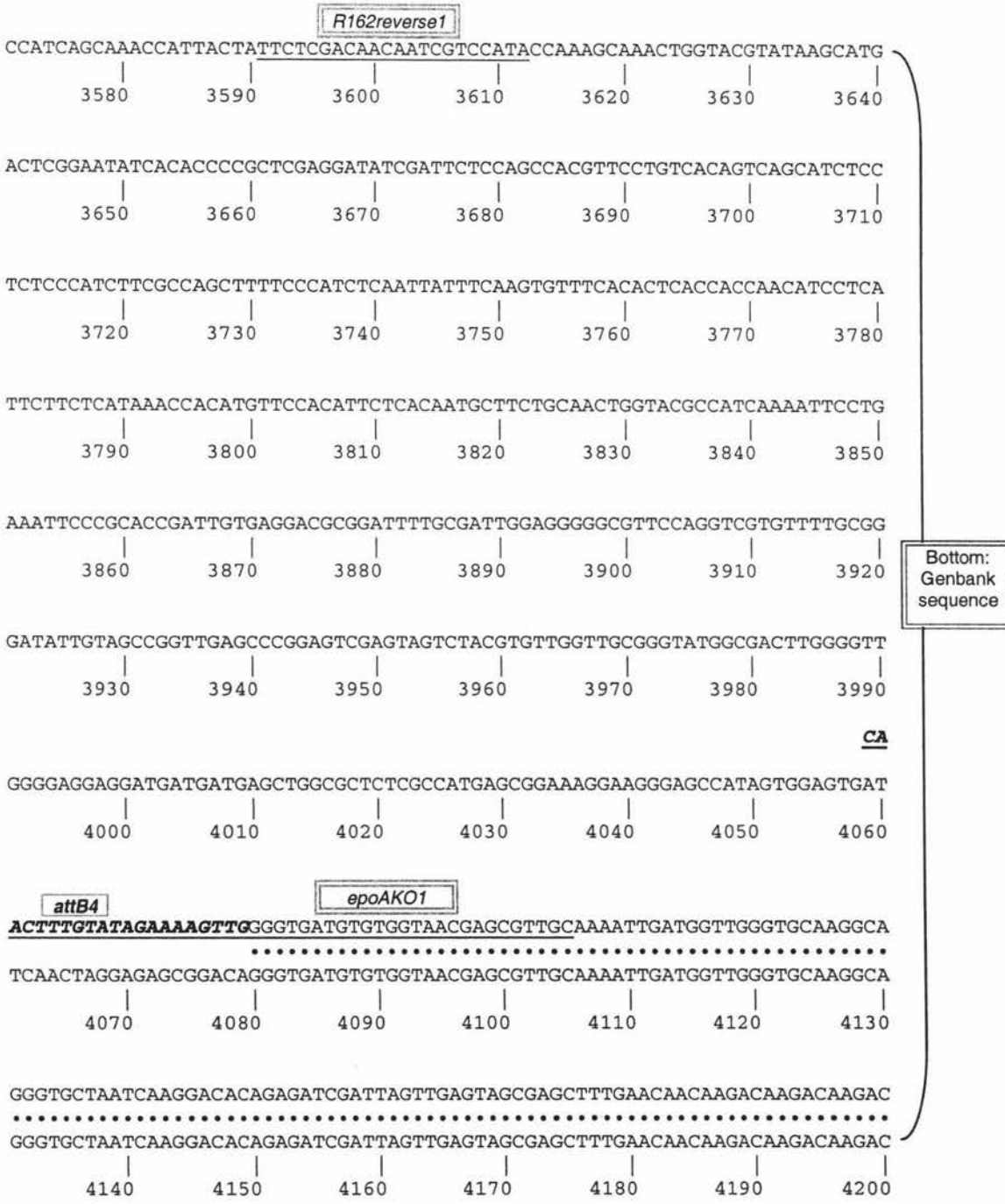
Top sequence: The junction sequence between the 5'or 3'element and *hph* cassette

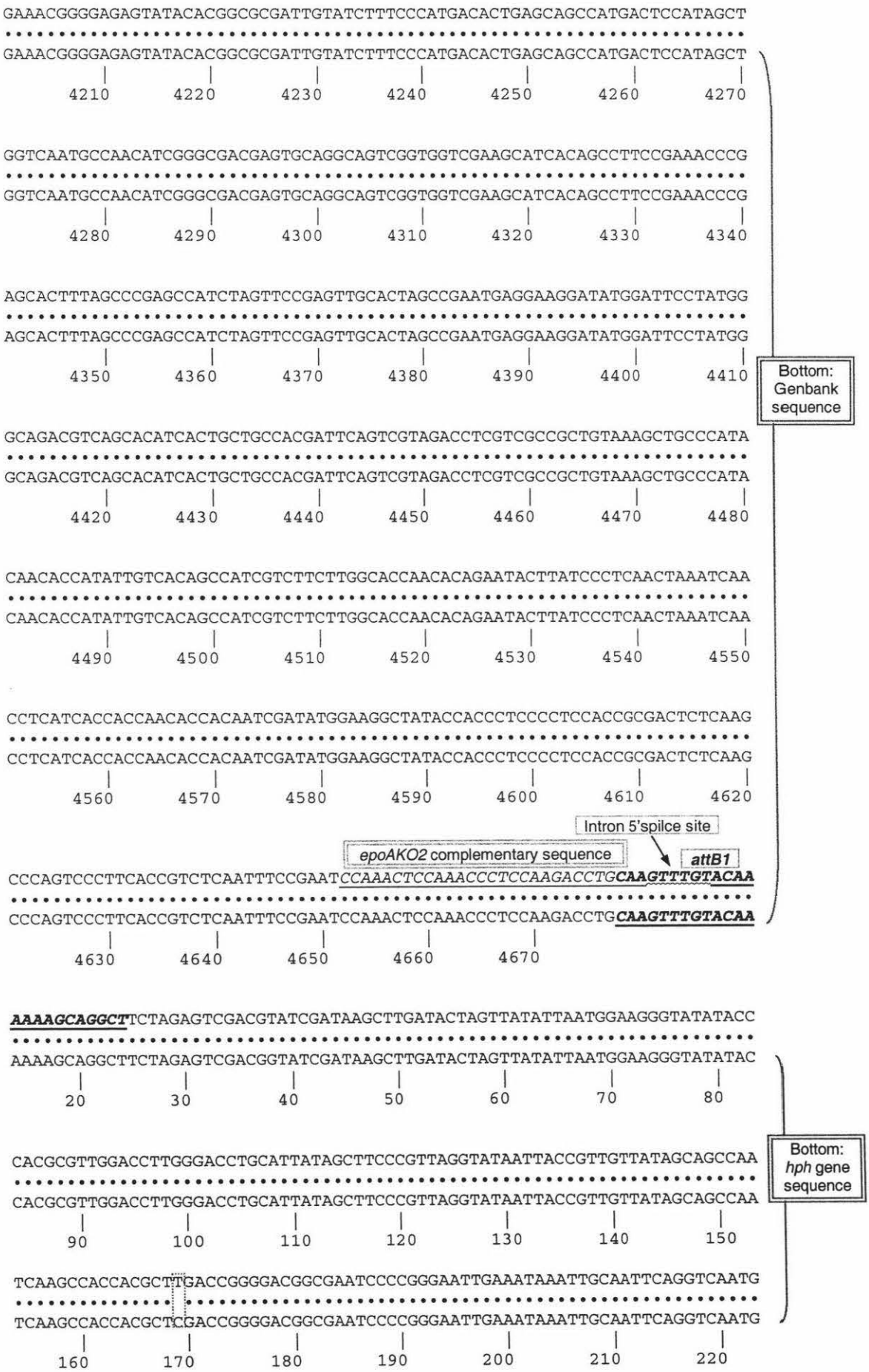
Bottom sequence: Genbank DQ149246 or *hph* gene sequence

: The primers names. The primers used to construct the 5' or 3' element and check the 5' or 3' region integration were underlined. The reverse primers are shown in the complementary sequence and underlined italics letters

attB sites(after LR recombination)

The sequence of the junction between the 5'element and *hph* gene casstte




GAAACGGGGAGAGTATACACGGCGCGATTGTATCTTTCCCATGACACTGAGCAGCCATGACTCCATAGCT
GAAACGGGGAGAGTATACACGGCGCGATTGTATCTTTCCCATGACACTGAGCAGCCATGACTCCATAGCT
4210 4220 4230 4240 4250 4260 4270
GGTCAATGCCAACATCGGGCGACGAGTGCAGGCAGTCGGTGGTCGAAGCATCACAGCCTTCCGAAACCCG
GGTCAATGCCAACATCGGGCGACGAGTGCAGGCAGTCGGTGGTCGAAGCATCACAGCCTTCCGAAACCCG
4280 4290 4300 4310 4320 4330 4340
AGCACTTTAGCCCGAGCCATCTAGTTCCGAGTTGCACTAGCCGAATGAGGAAGGATATGGATTCCTATGG
AGCACTTTAGCCCGAGCCATCTAGTTCCGAGTTGCACTAGCCGAATGAGGAAGGATATGGATTCCTATGG
4350 4360 4370 4380 4390 4400 4410
Bottom: Genbank sequence
GCAGACGTCAGCACATCACTGCTGCCACGATTCAGTCGTAGACCTCGTCGCCGCTGTAAAGCTGCCCATA
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4420 4430 4440 4450 4460 4470 4480
CAACACCATATTGTCACAGCCATCGTCTTCTTGGCACCAACACAGAATACTTATCCCTCAACTAAATCAA
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4490 4500 4510 4520 4530 4540 4550
CCTCATCACCACCAACACCACAATCGATATGGAAGGCTATACCACCCTCCCCTCCACCGCGACTCTCAAG
CCTCATCACCACCAACACCACAATCGATATGGAAGGCTATACCACCCTCCCCTCCACCGCGACTCTCAAG
4560 4570 4580 4590 4600 4610 4620
Intron 5'spilce site
epoAKO2 complementary sequence
attB1
CCCAGTCCCTTCACCGTCTCAATTTCCGAATCCAAACTCCAAACCCTCCAAGACCTGCAAGTTTGTACAA
CCCAGTCCCTTCACCGTCTCAATTTCCGAATCCAAACTCCAAACCCTCCAAGACCTGCAAGTTTGTACAA
4630 4640 4650 4660 4670
AAAAGCAGGCTTCTAGAGTCGACGTATCGATAAGCTTGATACTAGTTATATTAATGGAAGGGTATATACC
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20 30 40 50 60 70 80
Bottom: hph gene sequence
CACGCGTTGGACCTTGGGACCTGCATTATAGCTTCCCGTTAGGTATAATTACCGTTGTTATAGCAGCCAA
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90 100 110 120 130 140 150
TCAAGCCACCACGCTTGACCGGGGACGGCGAATCCCCGGGAATTGAAATAAATTGCAATTCAGGTCAATG
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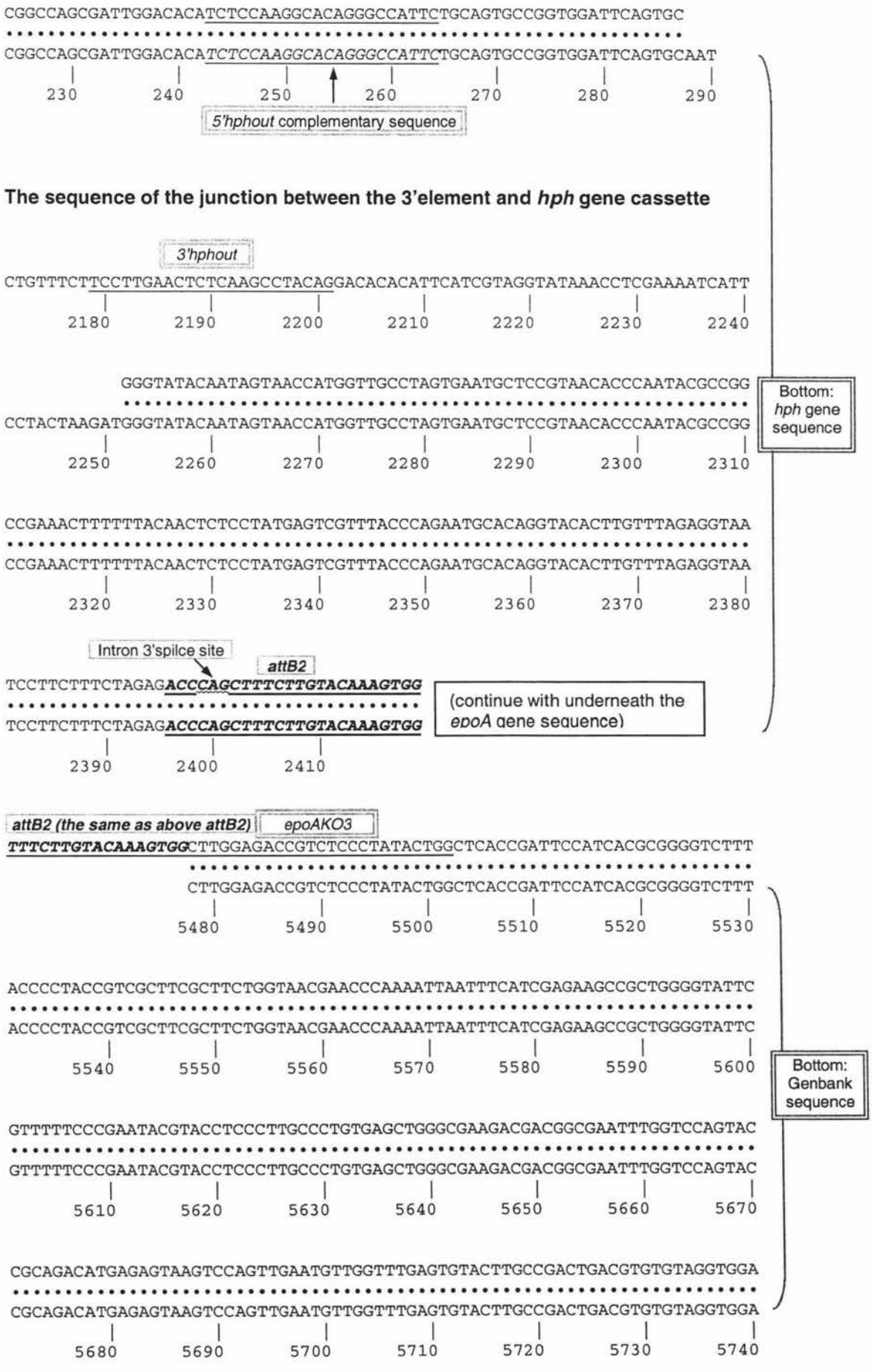
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CGGCCAGCGATTGGACACATCTCCAAGGCACAGGGCCATTCTGCAGTGCCGGTGGATTCAGTGCAAT
230 240 250 260 270 280 290
5'hphout complementary sequence
The sequence of the junction between the 3'element and hph gene cassette
3'hphout
CTGTTTCTTCCTTGAACTCTCAAGCCTACAGGACACACATTCATCGTAGGTATAAACCTCGAAAATCATT
2180 2190 2200 2210 2220 2230 2240
GGGTATACAATAGTAACCATGGTTGCCTAGTGAATGCTCCGTAACACCCAATACGCCGG
CCTACTAAGATGGGTATACAATAGTAACCATGGTTGCCTAGTGAATGCTCCGTAACACCCAATACGCCGG
2250 2260 2270 2280 2290 2300 2310
Bottom: hph gene sequence
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CCGAAACTTTTTTACAACTCTCCTATGAGTCGTTTACCCAGAATGCACAGGTACACTTGTTTAGAGGTAA
2320 2330 2340 2350 2360 2370 2380
Intron 3'spilce site
attB2
TCCTTCTTTCTAGAGACCCAGCTTTCTTGTACAAAGTGG
TCCTTCTTTCTAGAGACCCAGCTTTCTTGTACAAAGTGG
2390 2400 2410
(continue with underneath the epoA gene sequence)
attB2 (the same as above attB2)
epoAKO3
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CTTGGAGACCGTCTCCCTATACTGGCTCACCGATTCCATCACGCGGGGTCTTT
5480 5490 5500 5510 5520 5530
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5540 5550 5560 5570 5580 5590 5600
Bottom: Genbank sequence
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5610 5620 5630 5640 5650 5660 5670
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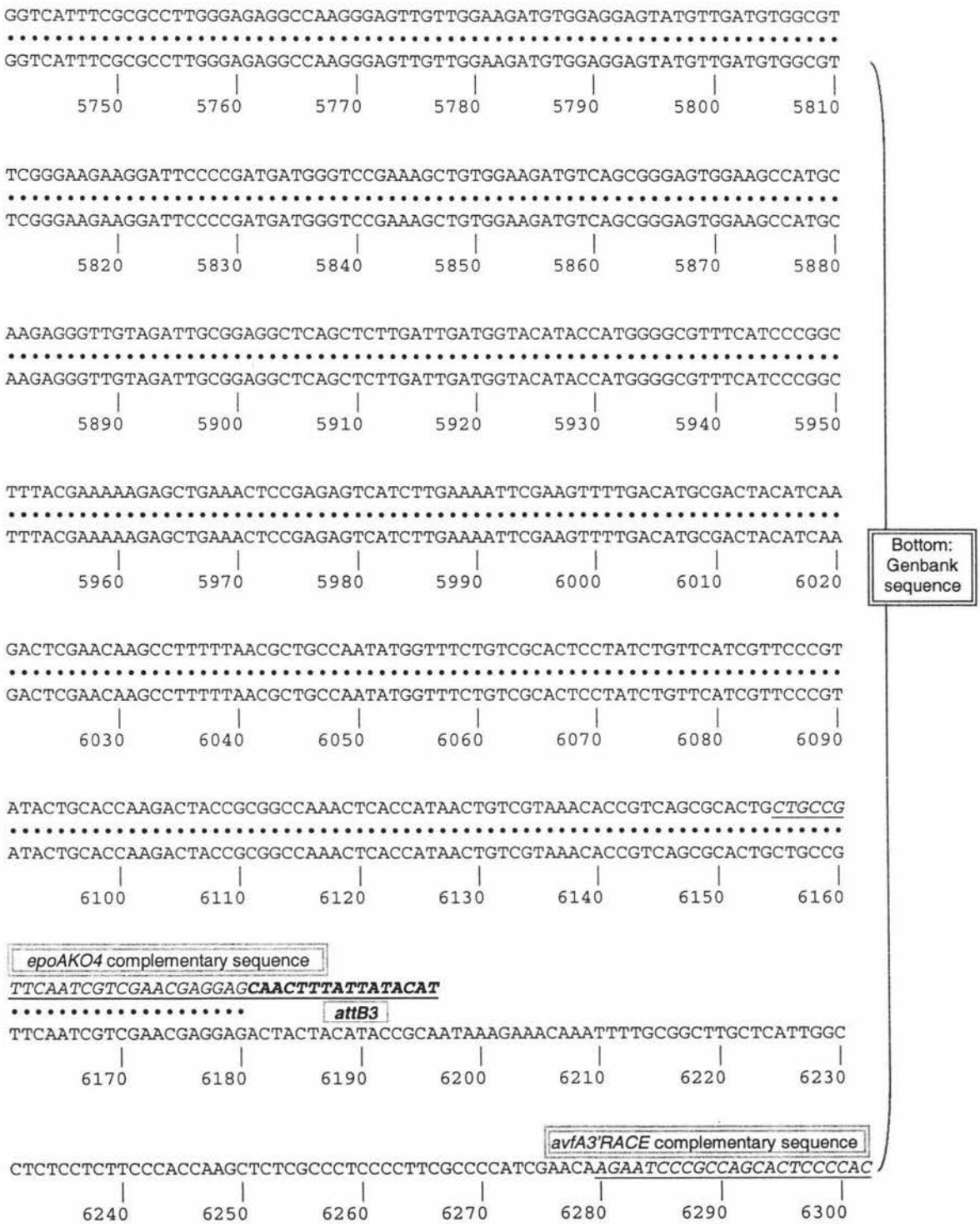
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5750 5760 5770 5780 5790 5800 5810
TCGGGAAGAAGGATTCCCCGATGATGGGTCCGAAAGCTGTGGAAGATGTCAGCGGGAGTGGAAGCCATGC
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5820 5830 5840 5850 5860 5870 5880
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5890 5900 5910 5920 5930 5940 5950
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5960 5970 5980 5990 6000 6010 6020
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6030 6040 6050 6060 6070 6080 6090
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ATACTGCACCAAGACTACCGCGGCCAAACTCACCATAACTGTCGTAAACACCGTCAGCGCACTGCTGCCG
6100 6110 6120 6130 6140 6150 6160
epoAKO4 complementary sequence
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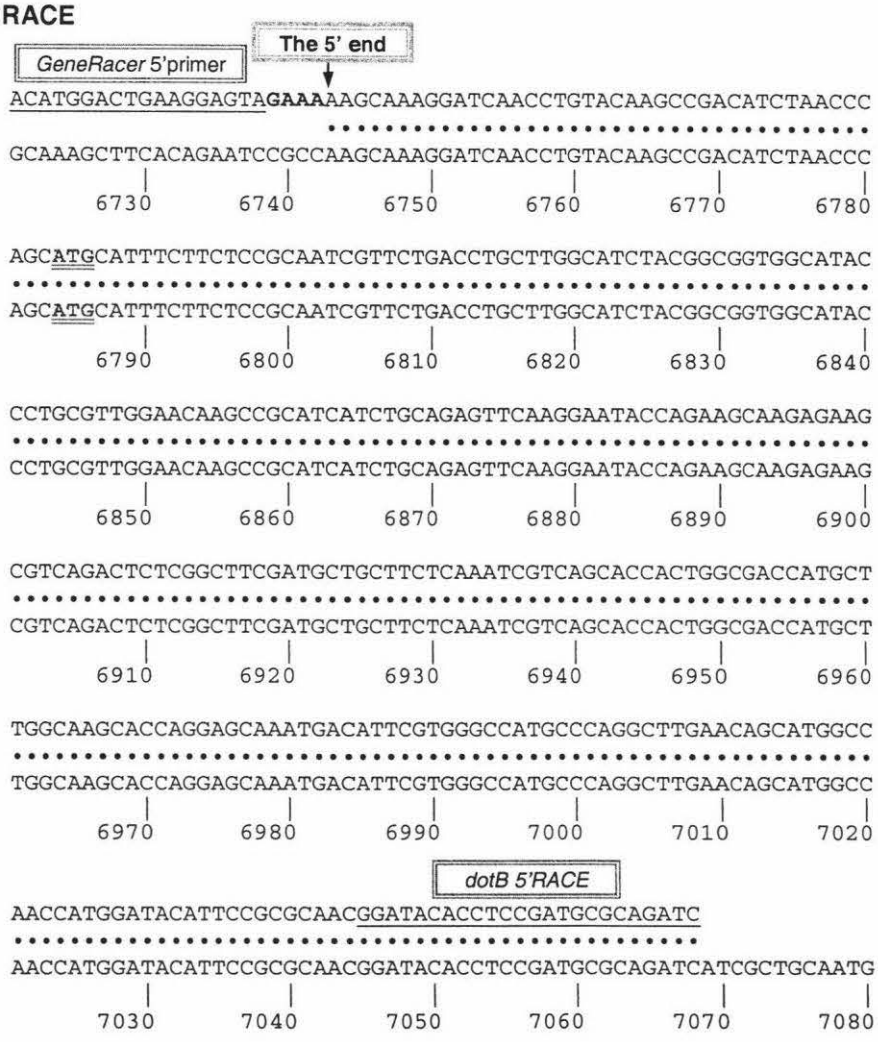
attB3
TTCAATCGTCGAACGAGGAGACTACTACATACCGCAATAAAGAAACAAATTTTGCGGCTTGCTCATTGGC
6170 6180 6190 6200 6210 6220 6230
avfA3'RACE complementary sequence
CTCTCCTCTTCCCACCAAGCTCTCGCCCTCCCCTTCGCCCCATCGAACAAGAATCCCGCCAGCACTCCCCAC
6240 6250 6260 6270 6280 6290 6300
Bottom: Genbank sequence

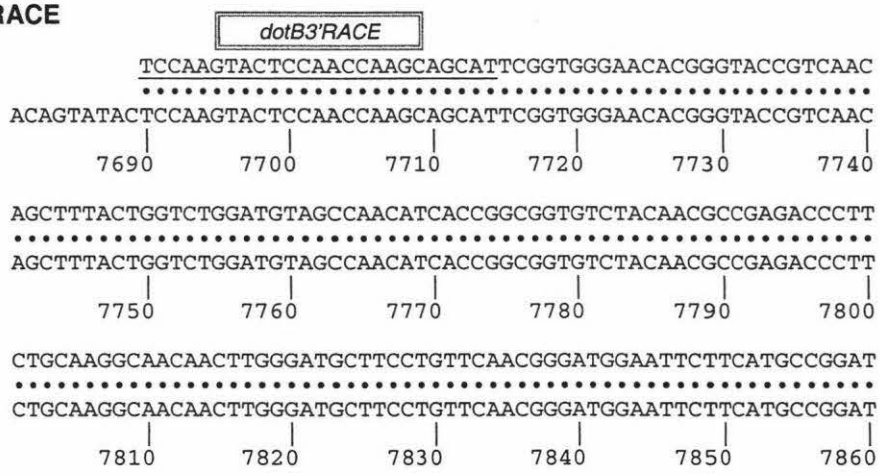
Appendix IV RACE SEQUENCES

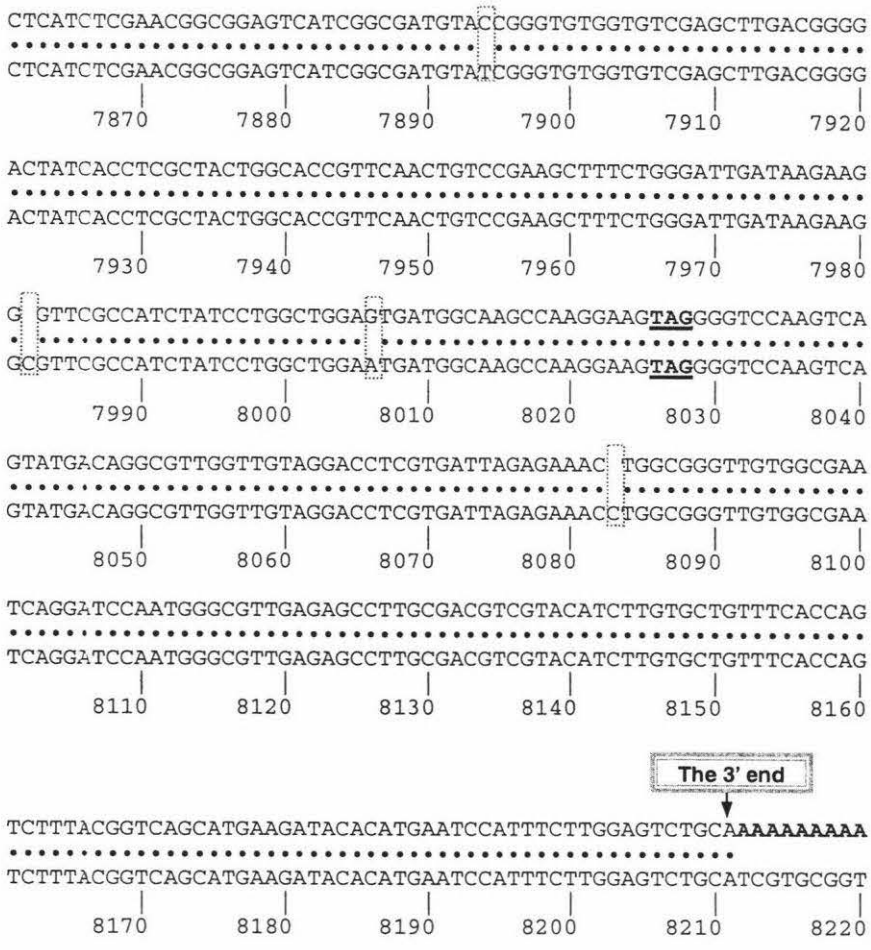
Top sequence: the 5' or 3'RACE sequence ofdothistromin genes; **Bottom sequence**: dothistromin genomic DNA sequences(Genbank: AF448056 or DQ149246). **The primers** using to amplify the RACE sequence are underlined. **The primer names** and the **5' or 3'UTR end** are labelled in the text box.
⬚ : mismatch. ATG: translation start codon; **TAG**: translation stop codon.

5' *dotB* RACE



3'*dotB* RACE

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CTCATCTCGAACGGCGGAGTCATCGGCGATGTATCGGGTGTGGTGTCGAGCTTGACGGGG
7870 7880 7890 7900 7910 7920
ACTATCACCTCGCTACTGGCACCGTTCAACTGTCCGAAGCTTTCTGGGATTGATAAGAAG
ACTATCACCTCGCTACTGGCACCGTTCAACTGTCCGAAGCTTTCTGGGATTGATAAGAAG
7930 7940 7950 7960 7970 7980
GGTTCGCCATCTATCCTGGCTGGAGTGATGGCAAGCCAAGGAAGTAGGGGTCCAAGTCA
GCGTTCGCCATCTATCCTGGCTGGAATGATGGCAAGCCAAGGAAGTAGGGGTCCAAGTCA
7990 8000 8010 8020 8030 8040
GTATGACAGGCGTTGGTTGTAGGACCTCGTGATTAGAGAAACTGGCGGGTTGTGGCGAA
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8050 8060 8070 8080 8090 8100
TCAGGATCCAATGGGCGTTGAGAGCCTTGCGACGTCGTACATCTTGTGCTGTTTCACCAG
TCAGGATCCAATGGGCGTTGAGAGCCTTGCGACGTCGTACATCTTGTGCTGTTTCACCAG
8110 8120 8130 8140 8150 8160
The 3' end
TCTTTACGGTCAGCATGAAGATACACATGAATCCATTTCTTGGAGTCTGCAAAAAAAAAA
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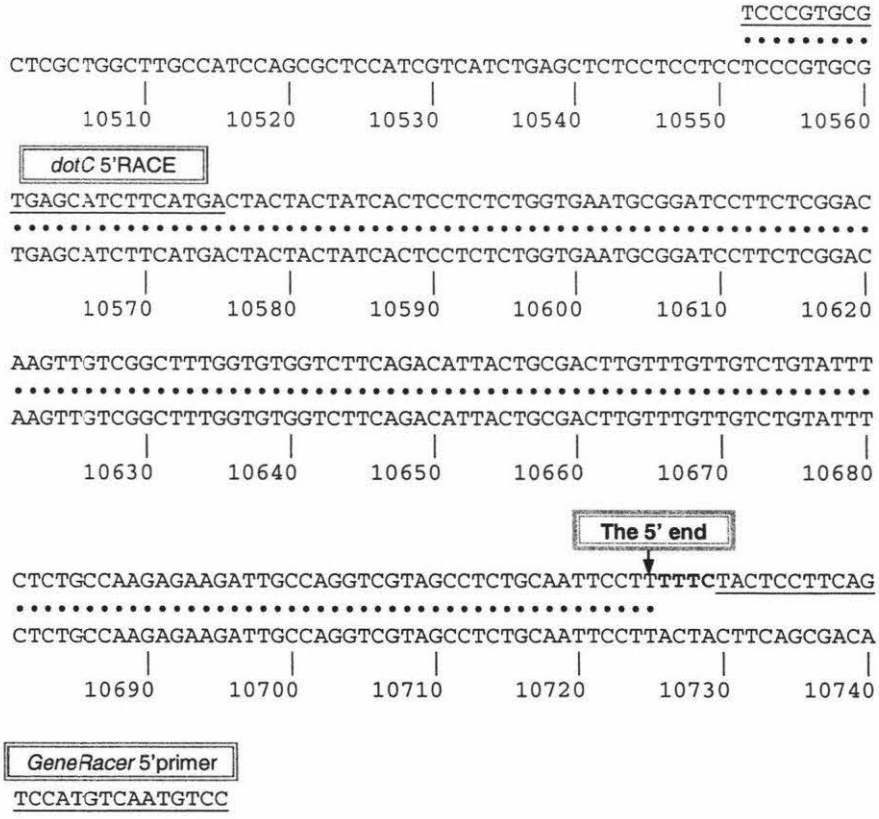
8170 8180 8190 8200 8210 8220

***dotC* 5' RACE**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10510 10520 10530 10540 10550 10560
dotC 5'RACE
TGAGCATCTTCATGACTACTACTATCACTCCTCTCTGGTGAATGCGGATCCTTCTCGGAC
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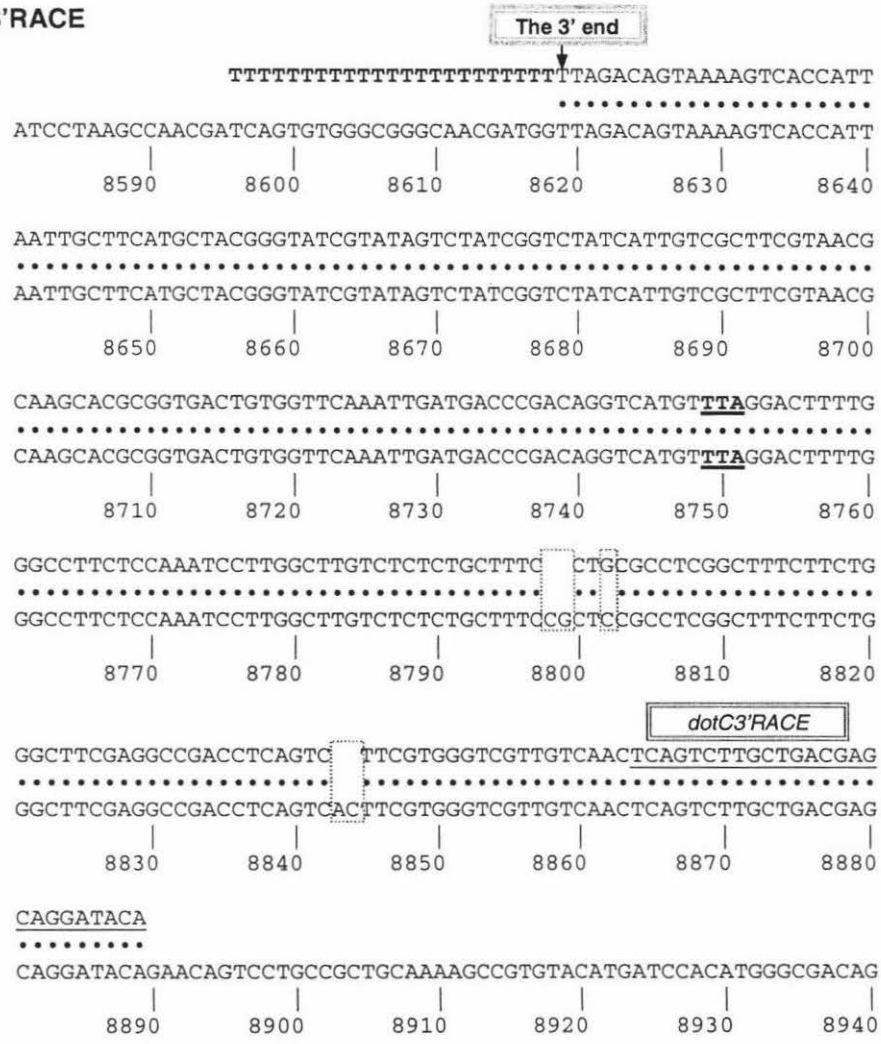
10570 10580 10590 10600 10610 10620
AAGTTGTCGGCTTTGGTGTGGTCTTCAGACATTACTGCGACTTGTTTGTTGTCTGTATTT
AAGTTGTCGGCTTTGGTGTGGTCTTCAGACATTACTGCGACTTGTTTGTTGTCTGTATTT
10630 10640 10650 10660 10670 10680
The 5' end
CTCTGCCAAGAGAAGATTGCCAGGTCGTAGCCTCTGCAATTCCTTTTTCTACTCCTTCAG
CTCTGCCAAGAGAAGATTGCCAGGTCGTAGCCTCTGCAATTCCTTACTACTTCAGCGACA
10690 10700 10710 10720 10730 10740
GeneRacer 5'primer
TCCATGTCAATGTCC

***dotC* 3'RACE**



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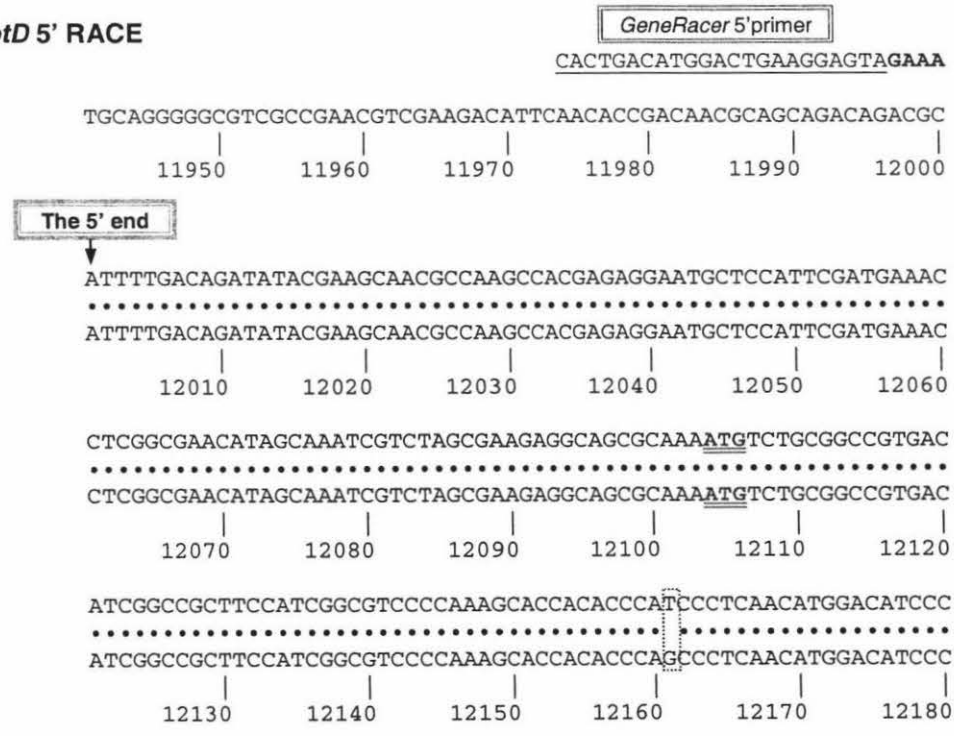

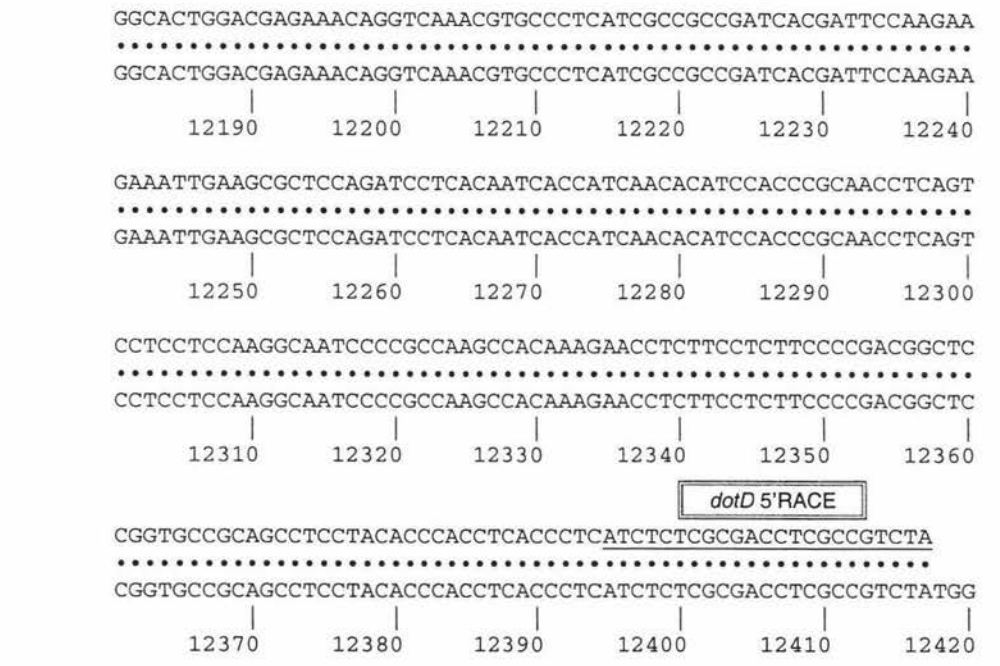
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GAAATTGAAGCGCTCCAGATCCTCACAATCACCATCAACACATCCACCCGCAACCTCAGT
GAAATTGAAGCGCTCCAGATCCTCACAATCACCATCAACACATCCACCCGCAACCTCAGT
12250 12260 12270 12280 12290 12300
CCTCCTCCAAGGCAATCCCCGCCAAGCCACAAAGAACCTCTTCCTCTTCCCCGACGGCTC
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12310 12320 12330 12340 12350 12360
dotD 5'RACE
CGGTGCCGCAGCCTCCTACACCCACCTCACCCTCATCTCTCGCGACCTCGCCGTCTA
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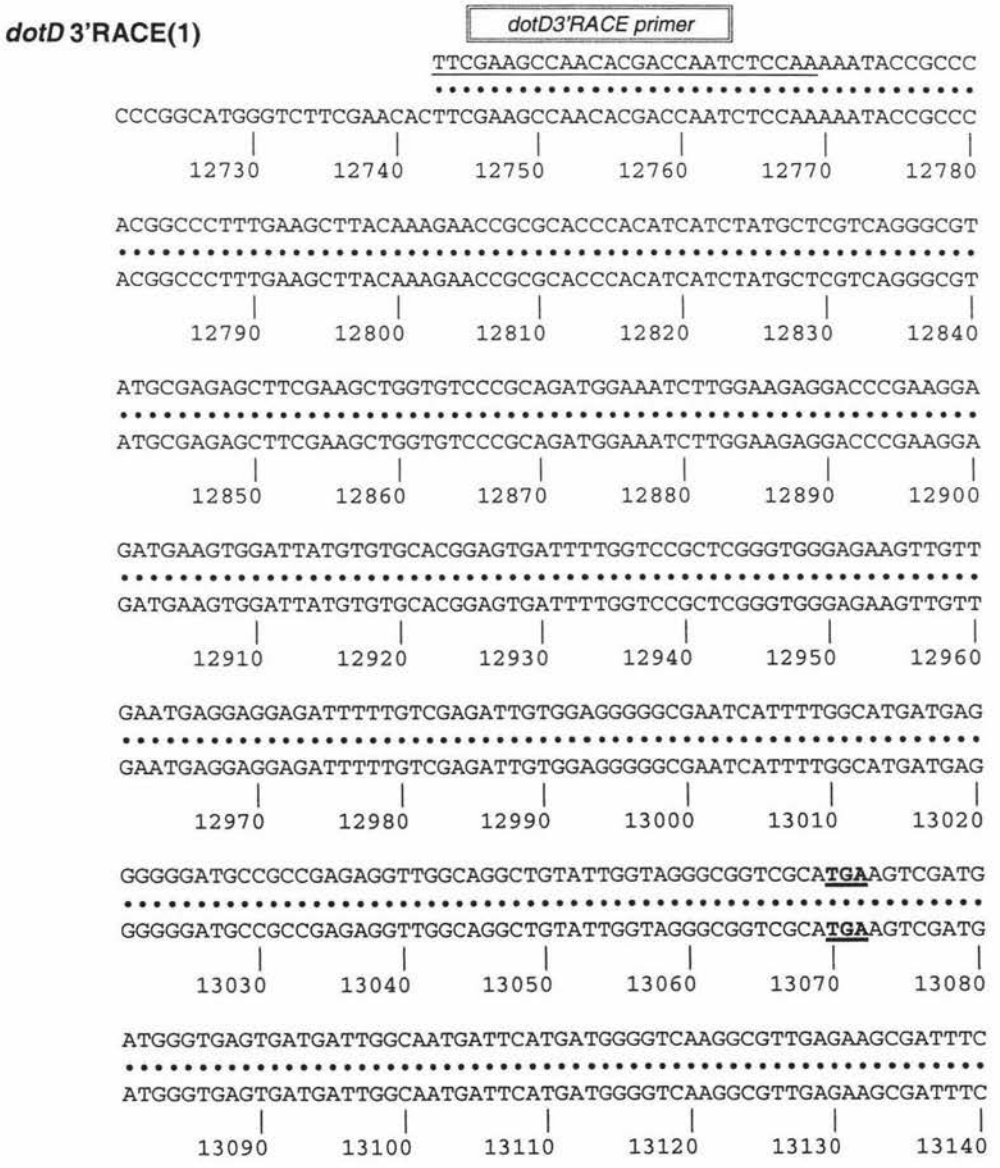
12370 12380 12390 12400 12410 12420

dotD 3'RACE(1)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12790 12800 12810 12820 12830 12840
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12850 12860 12870 12880 12890 12900
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12910 12920 12930 12940 12950 12960
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12970 12980 12990 13000 13010 13020
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13030 13040 13050 13060 13070 13080
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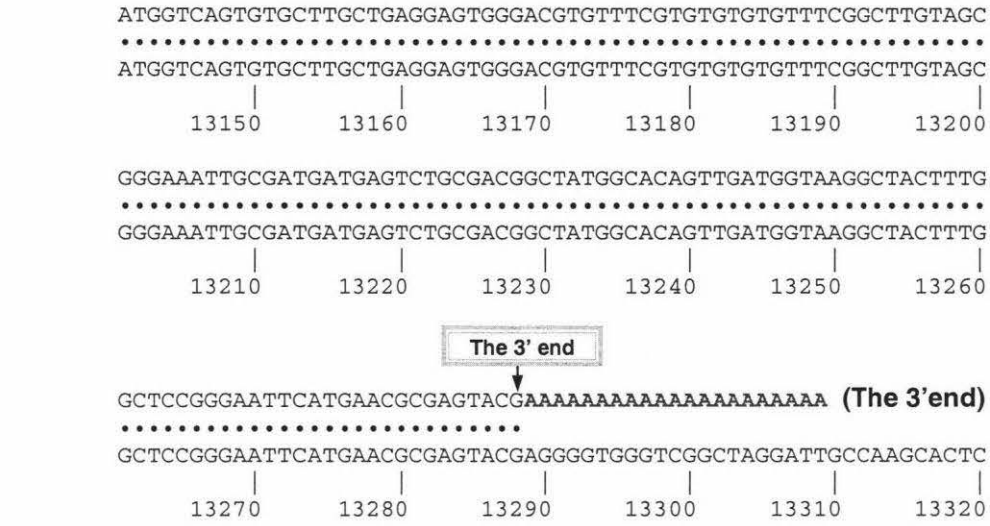
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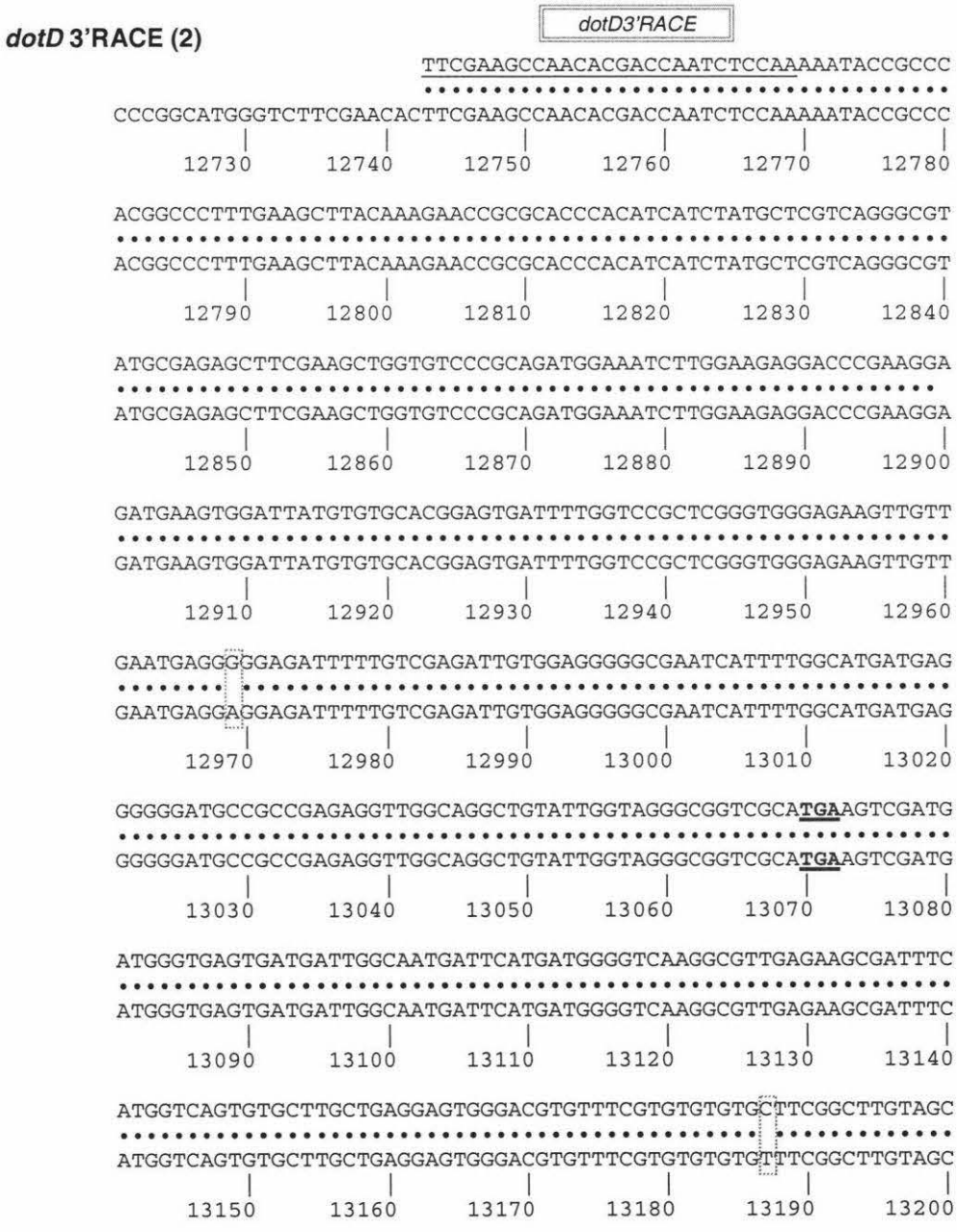
13150 13160 13170 13180 13190 13200
GGGAAATTGCGATGATGAGTCTGCGACGGCTATGGCACAGTTGATGGTAAGGCTACTTTG
GGGAAATTGCGATGATGAGTCTGCGACGGCTATGGCACAGTTGATGGTAAGGCTACTTTG
13210 13220 13230 13240 13250 13260
The 3' end
GCTCCGGGAATTCATGAACGCGAGTACGAAAAAAAAAAAAAAAAAAAA (The 3'end)
GCTCCGGGAATTCATGAACGCGAGTACGAGGGGTGGGTCGGCTAGGATTGCCAAGCACTC
13270 13280 13290 13300 13310 13320

***dotD* 3'RACE (2)**


dotD3'RACE
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ACGGCCCTTTGAAGCTTACAAAGAACCGCGCACCCACATCATCTATGCTCGTCAGGGCGT
ACGGCCCTTTGAAGCTTACAAAGAACCGCGCACCCACATCATCTATGCTCGTCAGGGCGT
12790 12800 12810 12820 12830 12840
ATGCGAGAGCTTCGAAGCTGGTGTCCCGCAGATGGAAATCTTGGAAGAGGACCCGAAGGA
ATGCGAGAGCTTCGAAGCTGGTGTCCCGCAGATGGAAATCTTGGAAGAGGACCCGAAGGA
12850 12860 12870 12880 12890 12900
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12910 12920 12930 12940 12950 12960
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12970 12980 12990 13000 13010 13020
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13030 13040 13050 13060 13070 13080
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13090 13100 13110 13120 13130 13140
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13150 13160 13170 13180 13190 13200


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GCTCCGGGAATTCATGAACGCGAGTACGAGGGGTGGGTCGGCTAGGATTGCCAAGCACTC
13270 13280 13290 13300 13310 13320
The 3' end
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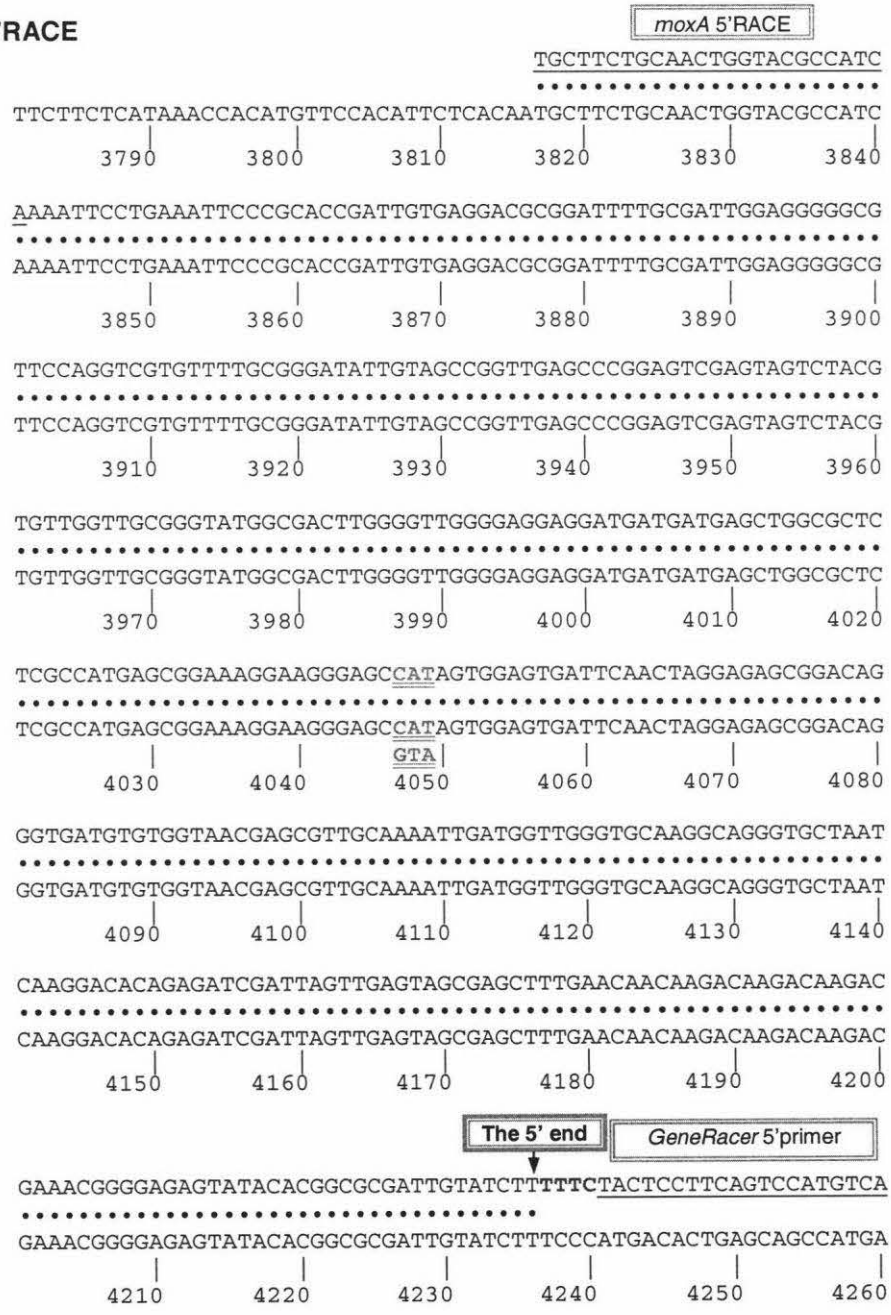
GTCAAGATCGAGAATTCCCGGGATCC
13330 13340

***moxA* 5'RACE**


moxA 5'RACE
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3850 3860 3870 3880 3890 3900
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3910 3920 3930 3940 3950 3960
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3970 3980 3990 4000 4010 4020
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TCGCCATGAGCGGAAAGGAAGGGAGCCATAGTGGAGTGATTCAACTAGGAGAGCGGACAG
GTA
4030 4040 4050 4060 4070 4080
GGTGATGTGTGGTAACGAGCGTTGCAAAATTGATGGTTGGGTGCAAGGCAGGGTGCTAAT
GGTGATGTGTGGTAACGAGCGTTGCAAAATTGATGGTTGGGTGCAAGGCAGGGTGCTAAT
4090 4100 4110 4120 4130 4140
CAAGGACACAGAGATCGATTAGTTGAGTAGCGAGCTTTGAACAACAAGACAAGACAAGAC
CAAGGACACAGAGATCGATTAGTTGAGTAGCGAGCTTTGAACAACAAGACAAGACAAGAC
4150 4160 4170 4180 4190 4200
The 5' end
GeneRacer 5'primer
GAAACGGGGAGAGTATACACGGCGCGATTGTATCTTTTTCTACTCCTTCAGTCCATGTCA
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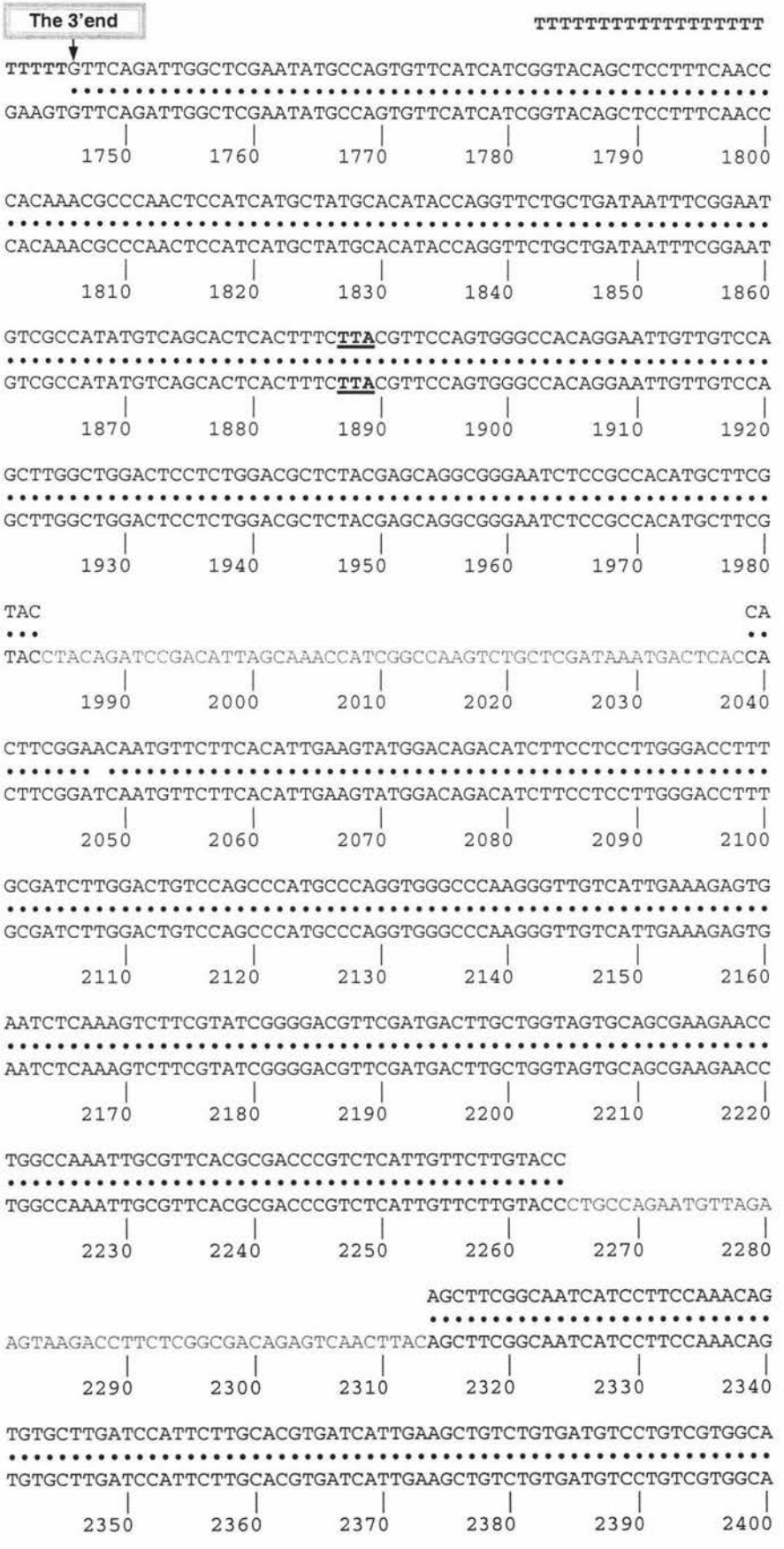
4210 4220 4230 4240 4250 4260

3'*moxA* RACE


The 3'end
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GAAGTGTTCAGATTGGCTCGAATATGCCAGTGTTCATCATCGGTACAGCTCCTTTCAACC
1750 1760 1770 1780 1790 1800
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CACAAACGCCCAACTCCATCATGCTATGCACATACCAGGTTCTGCTGATAATTTCGGAAT
1810 1820 1830 1840 1850 1860
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GTCGCCATATGTCAGCACTCACTTTCTTACGTTCCAGTGGGCCACAGGAATTGTTGTCCA
1870 1880 1890 1900 1910 1920
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GCTTGGCTGGACTCCTCTGGACGCTCTACGAGCAGGCGGGAATCTCCGCCACATGCTTCG
1930 1940 1950 1960 1970 1980
TAC
CA
TACCTACAGATCCGACATTAGCAAACCATCGGCCAAGTCTGCTCGATAAATGACTCACCA
1990 2000 2010 2020 2030 2040
CTTCGGAACAATGTTCTTCACATTGAAGTATGGACAGACATCTTCCTCCTTGGGACCTTT
CTTCGGATCAATGTTCTTCACATTGAAGTATGGACAGACATCTTCCTCCTTGGGACCTTT
2050 2060 2070 2080 2090 2100
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GCGATCTTGGACTGTCCAGCCCATGCCCAGGTGGGCCCAAGGGTTGTCATTGAAAGAGTG
2110 2120 2130 2140 2150 2160
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AATCTCAAAGTCTTCGTATCGGGGACGTTCGATGACTTGCTGGTAGTGCAGCGAAGAACC
2170 2180 2190 2200 2210 2220
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TGGCCAAATTGCGTTCACGCGACCCGTCTCATTGTTCTTGTACCCTGCCAGAATGTTAGA
2230 2240 2250 2260 2270 2280
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AGTAAGACCTTCTCGGCGACAGAGTCAACTTACAGCTTCGGCAATCATCCTTCCAAACAG
2290 2300 2310 2320 2330 2340
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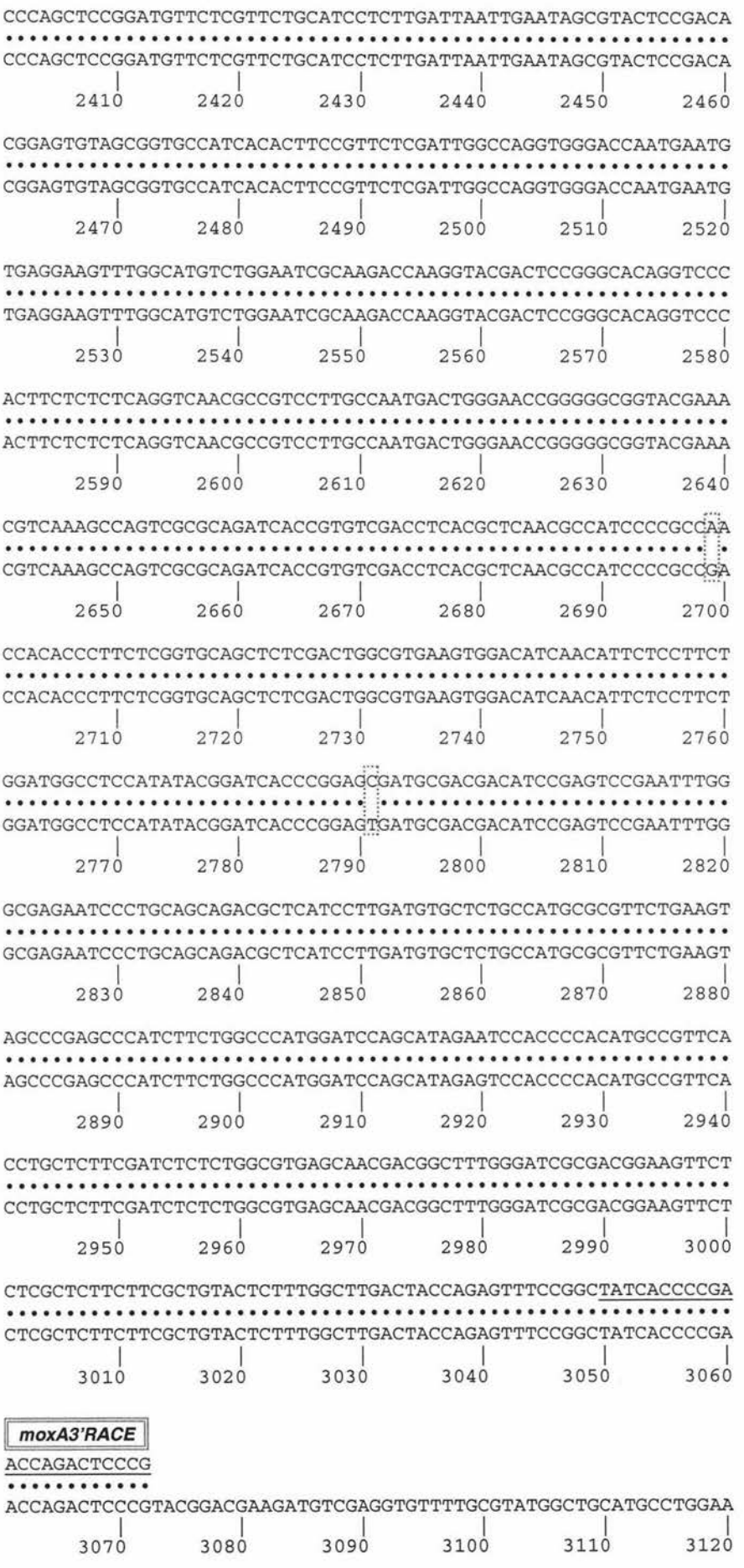
2350 2360 2370 2380 2390 2400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2410 2420 2430 2440 2450 2460
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2470 2480 2490 2500 2510 2520
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2770 2780 2790 2800 2810 2820
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2950 2960 2970 2980 2990 3000
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3010 3020 3030 3040 3050 3060
moxA3'RACE
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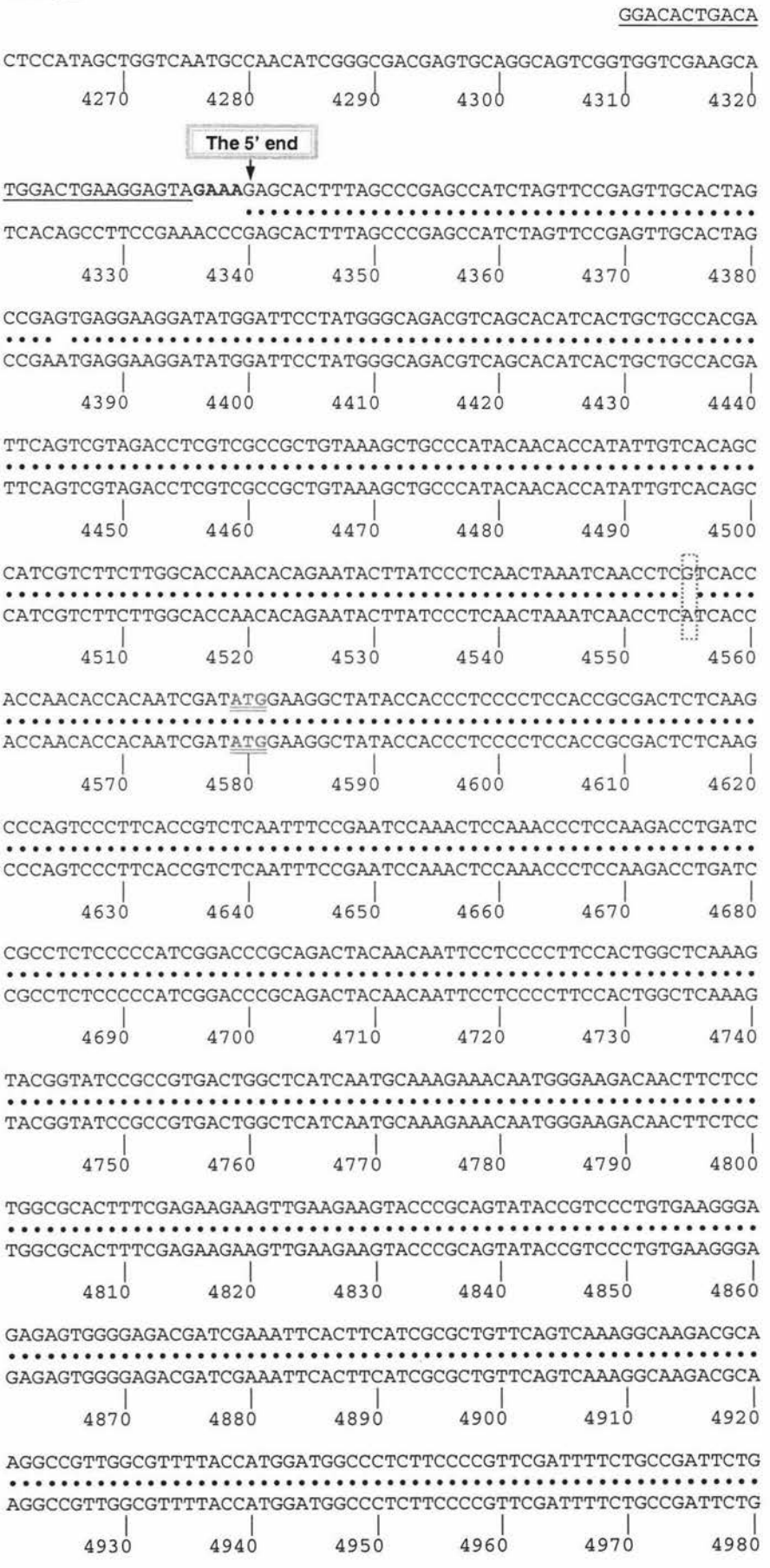
***epoA* 5' RACE**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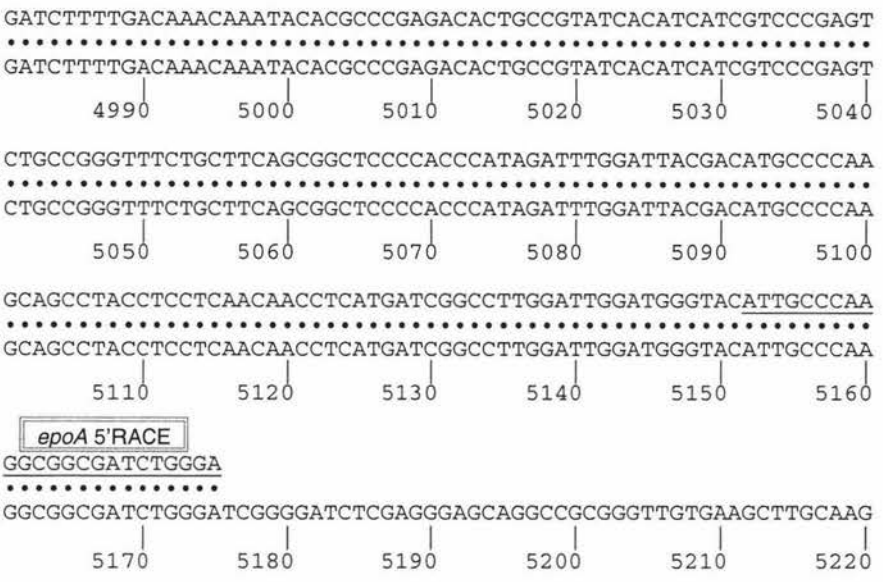
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5110 5120 5130 5140 5150 5160
epoA 5'RACE
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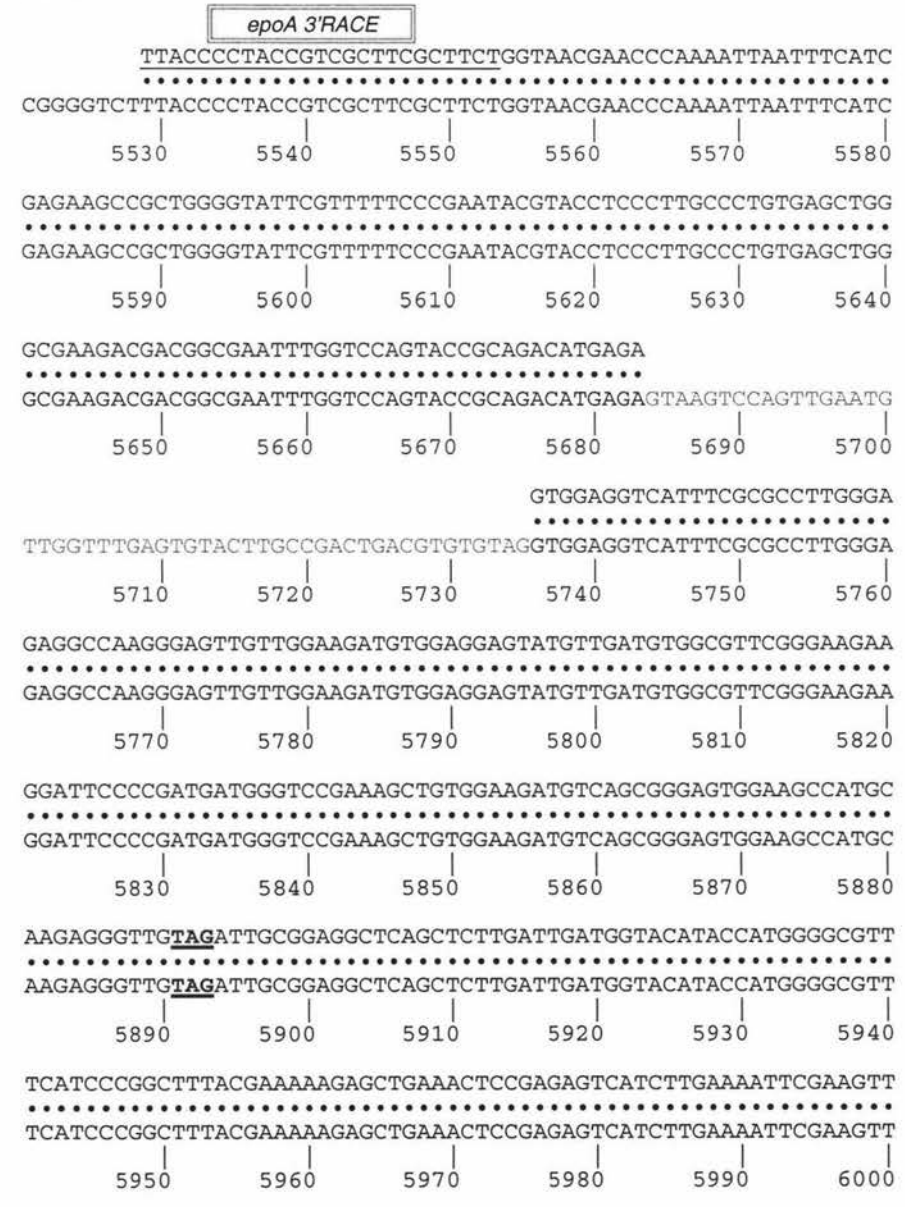
epoA 3' RACE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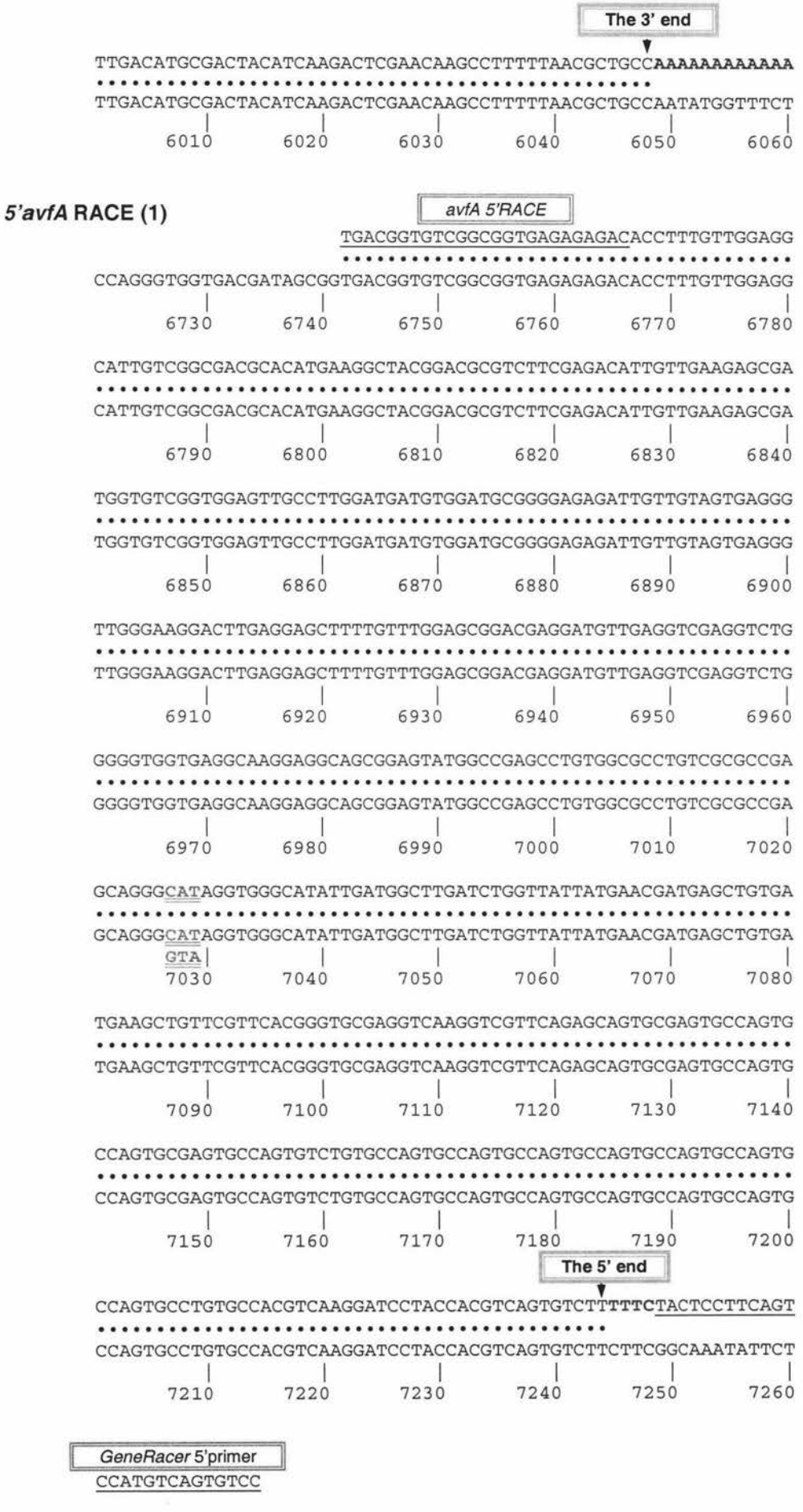
The 3' end
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5'avfA RACE (1)
avfA 5'RACE
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7090 7100 7110 7120 7130 7140
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7150 7160 7170 7180 7190 7200
The 5' end
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GeneRacer 5'primer
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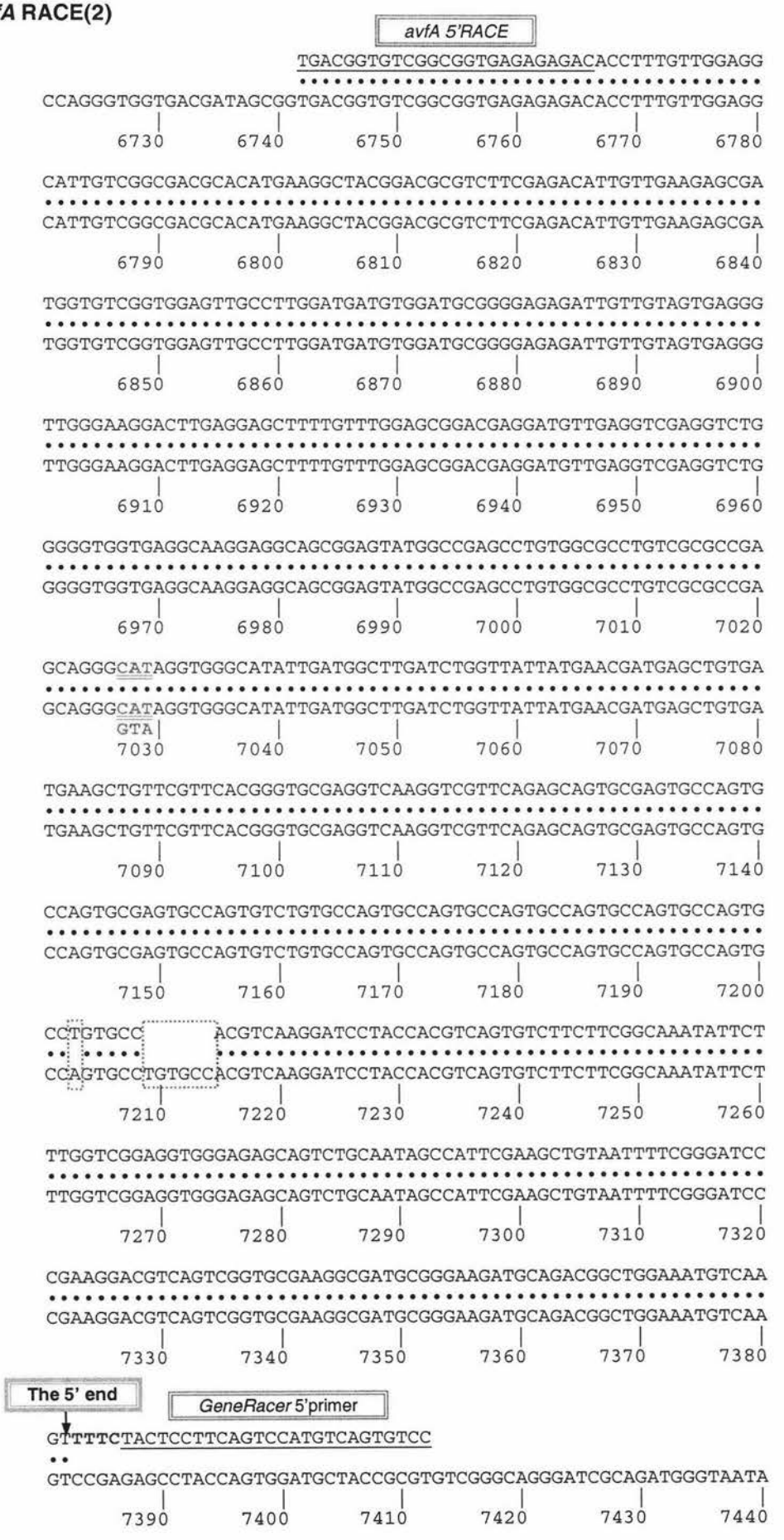
5'*avfA* RACE(2)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7210 7220 7230 7240 7250 7260
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7270 7280 7290 7300 7310 7320
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7330 7340 7350 7360 7370 7380
The 5' end
GeneRacer 5'primer
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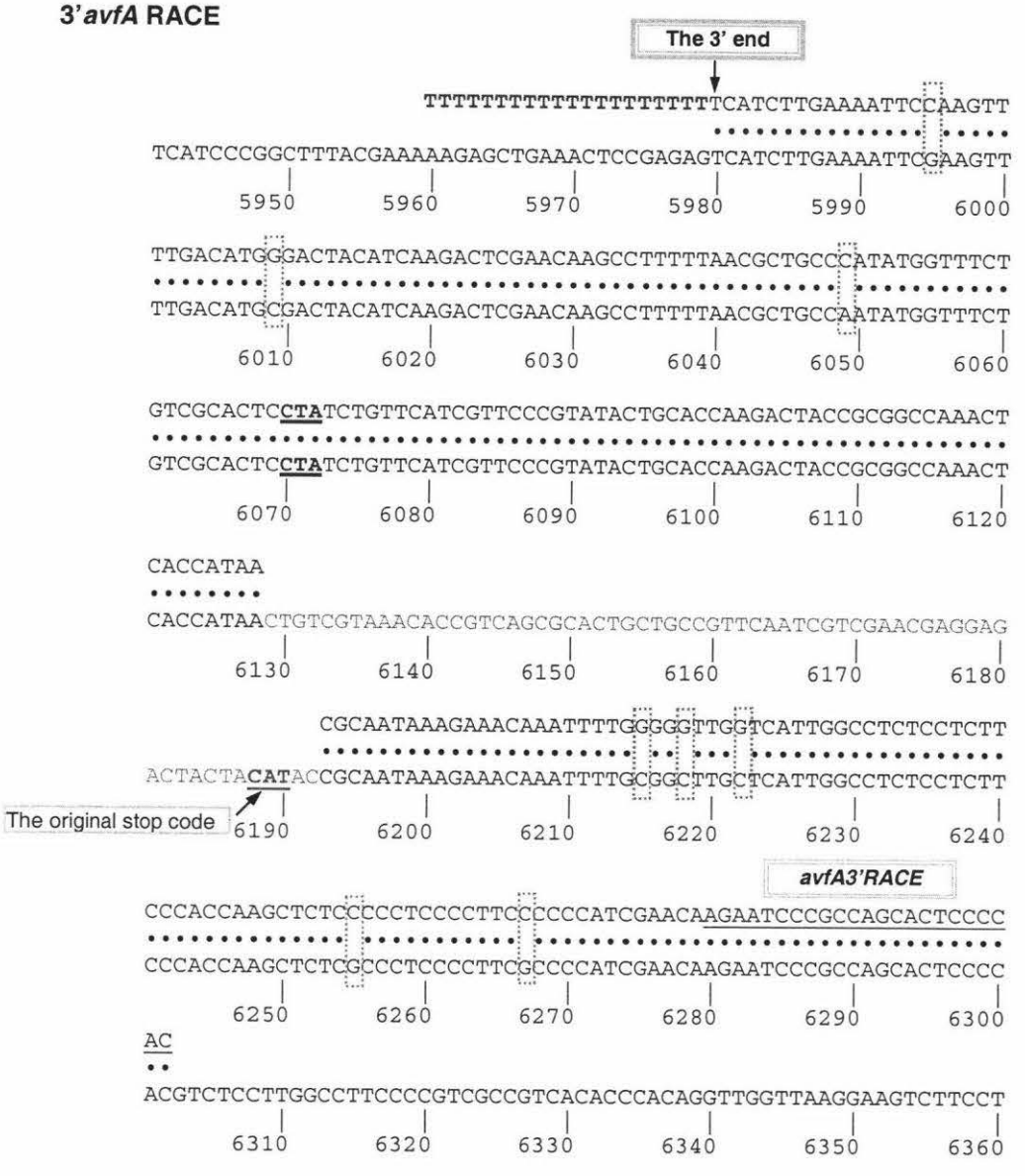
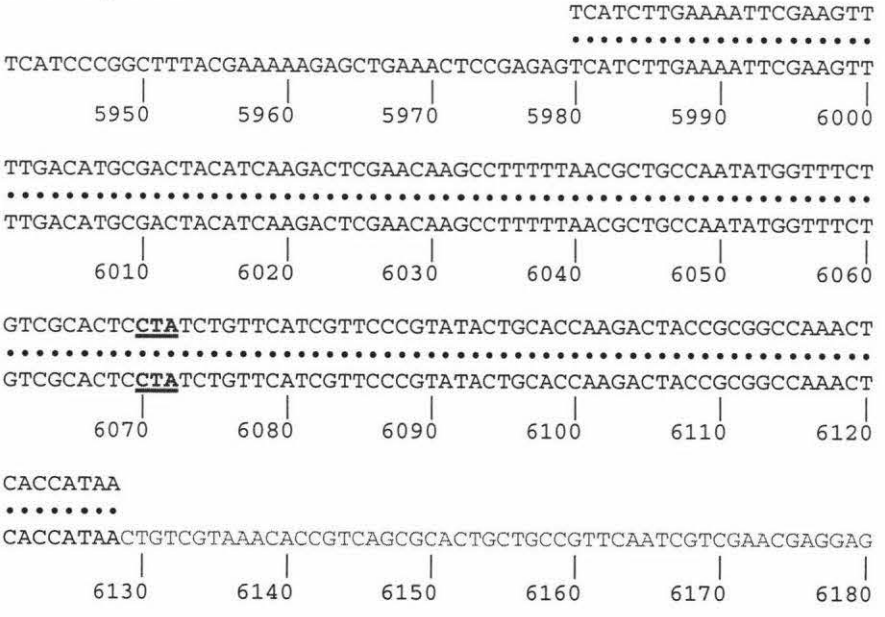
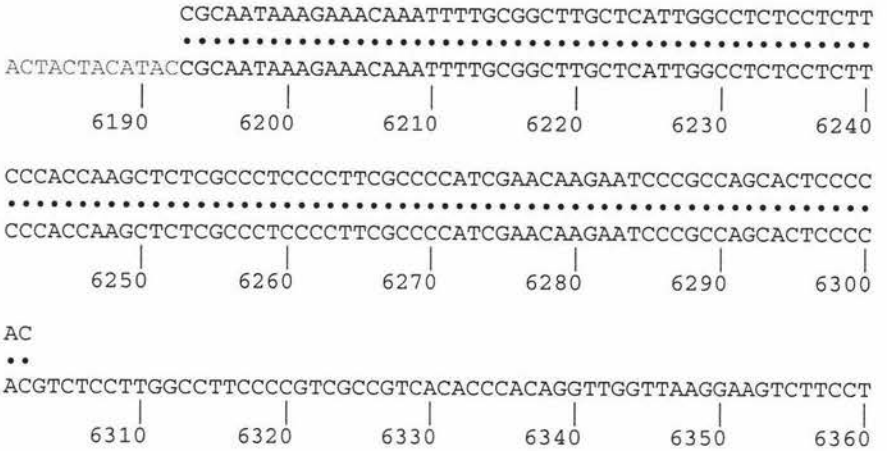
7390 7400 7410 7420 7430 7440

3'*avfA* RACE

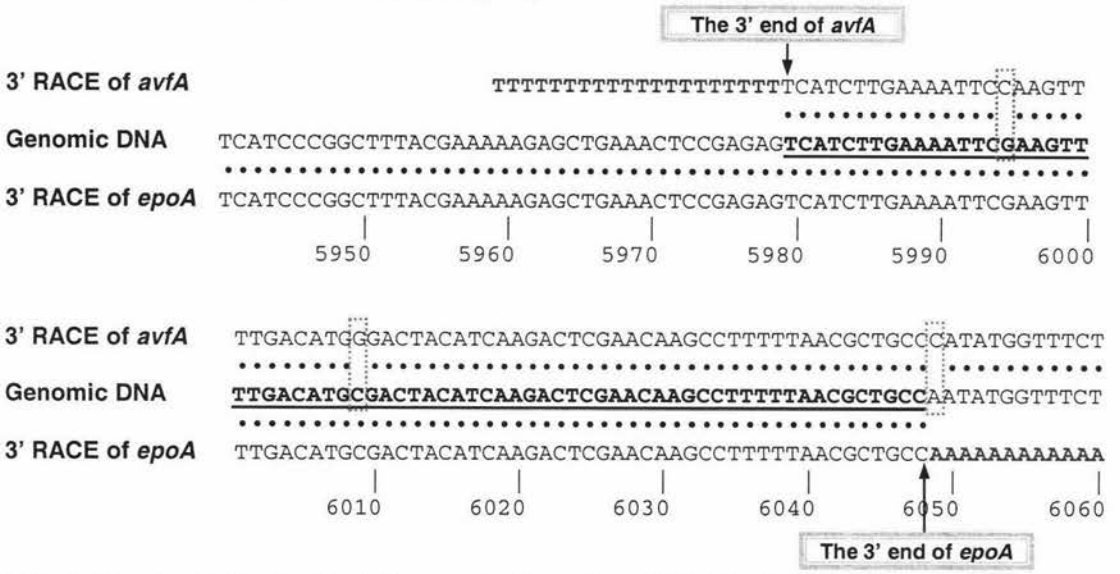


***avfA* RT-PCR sequence**



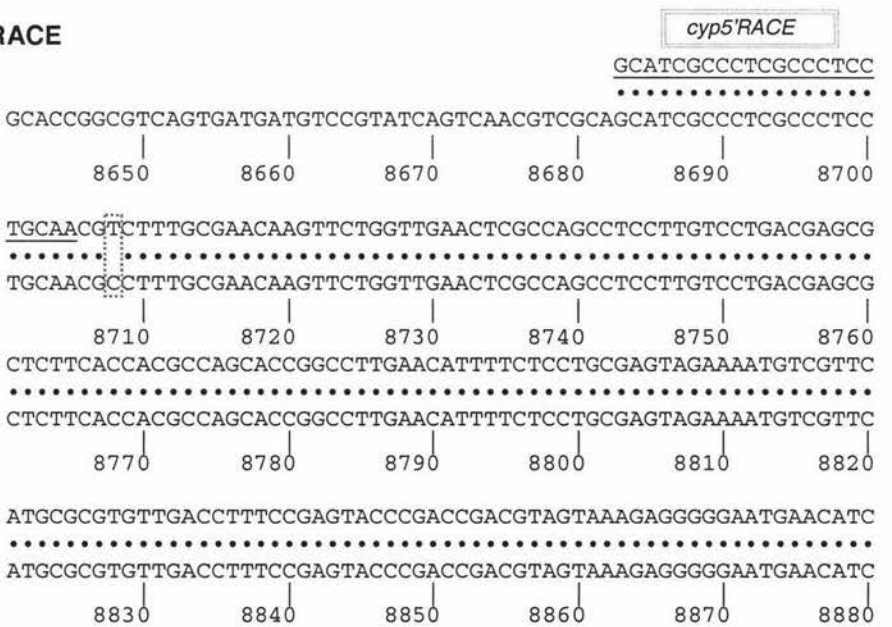


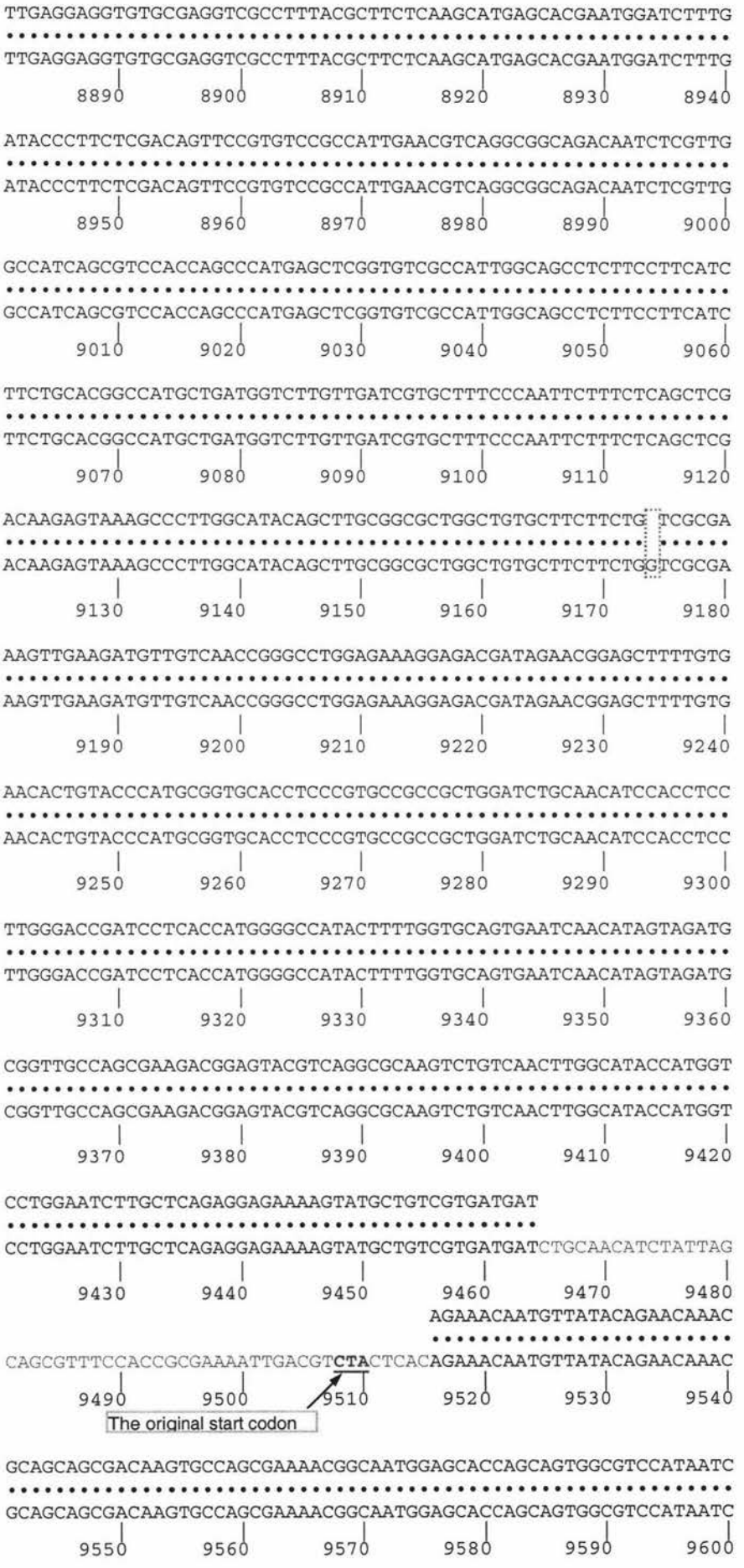
3'RACE of *avfA* and *epoA* overlapping region



* The bold and underlined region is the overlapping region of 3'RACE of *epoA* and *avfA*.

5'*cypA* RACE

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The original start codon
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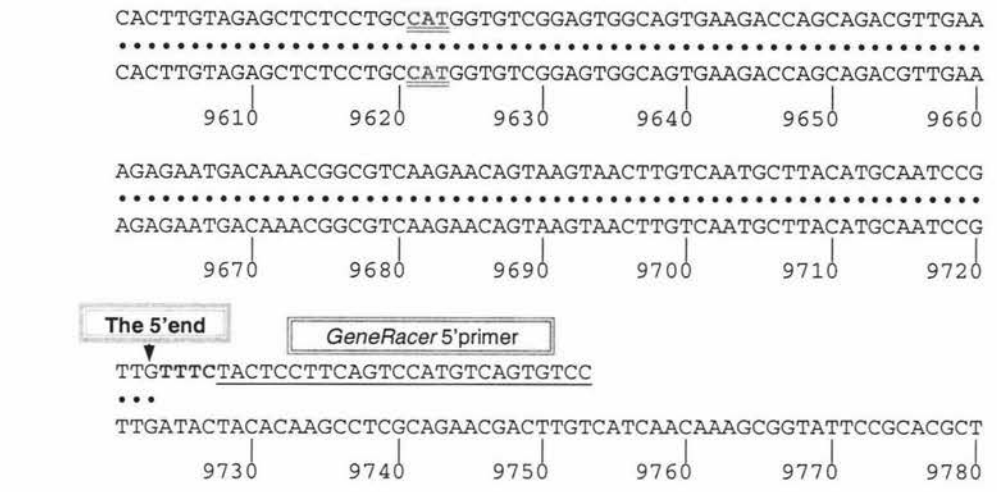
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The 5'end
GeneRacer 5'primer
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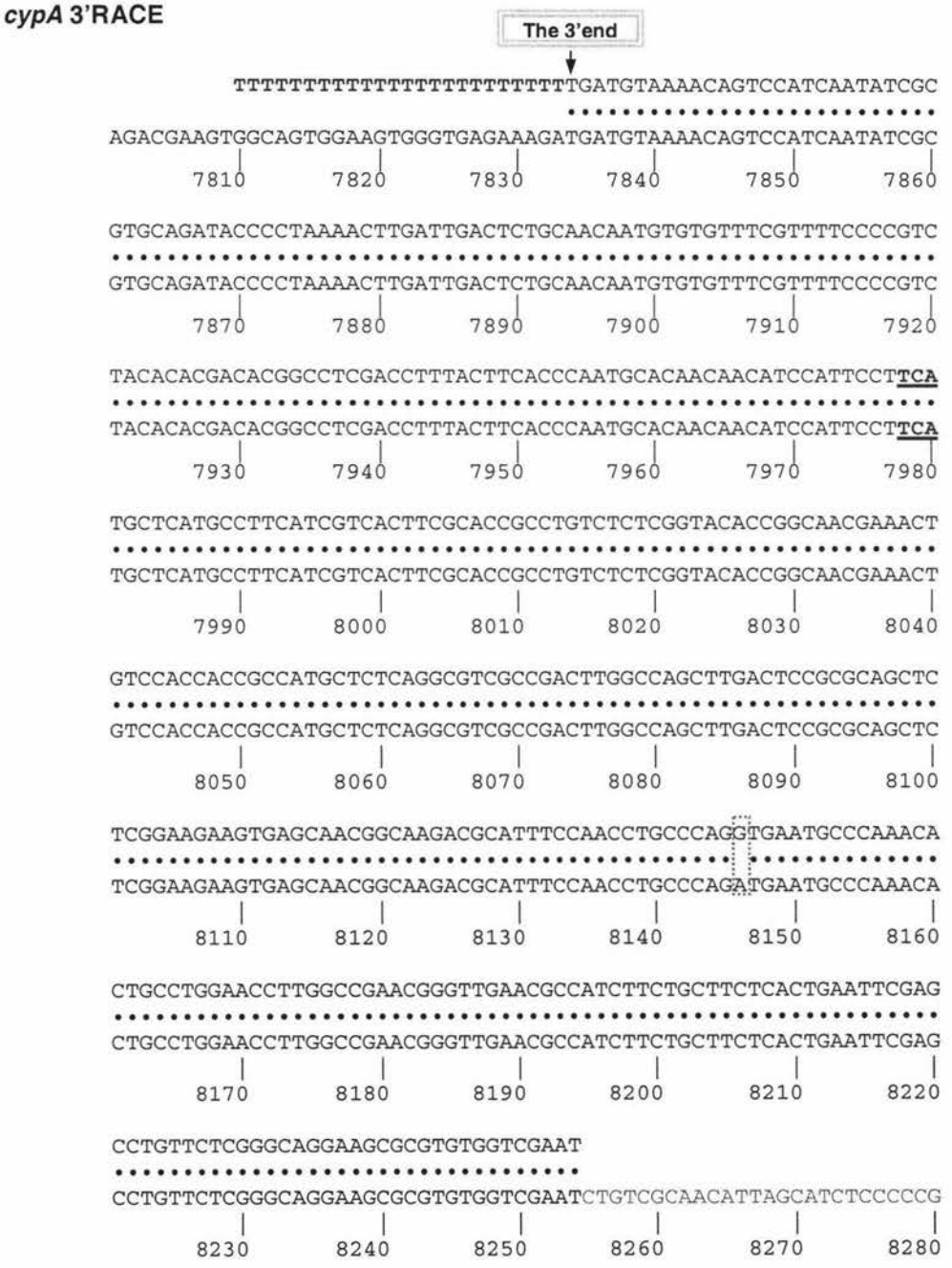
***cypA* 3'RACE**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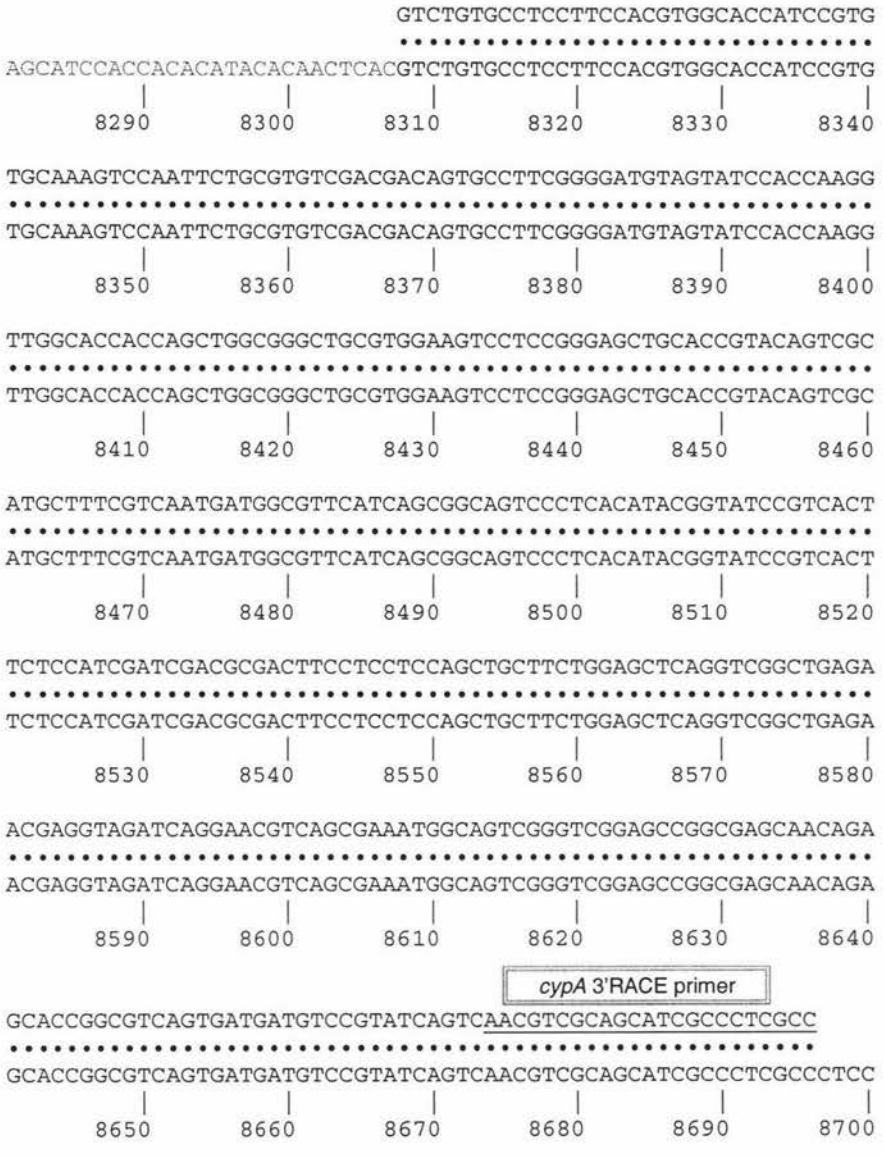
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cypA 3'RACE primer
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GCACCGGCGTCAGTGATGATGTCCGTATCAGTCAACGTCGCAGCATCGCCCTCGCCCTCC
8650 8660 8670 8680 8690 8700

Appendix V THE PUTATIVE REGULATORY BINDING MOTIFS THE IDENTIFIED DOTHISTROMIN GENES

tsp: transcriptional start site

t stop: transcriptional stop site

AbaA binging motifs

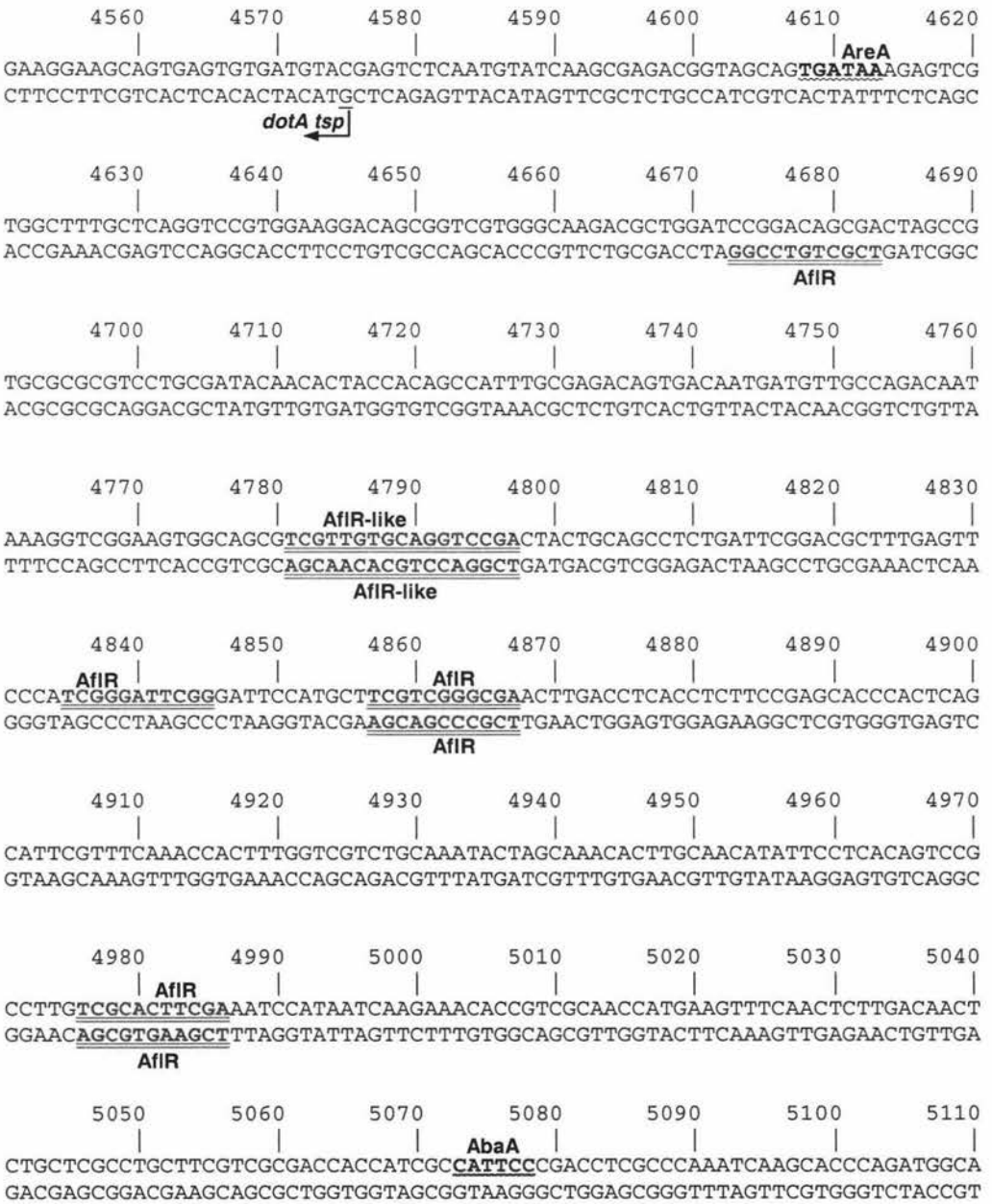
AflR ($TCGN_5CGR$) and AflR-like ($TCGN_{11}CGR$) binding motifs

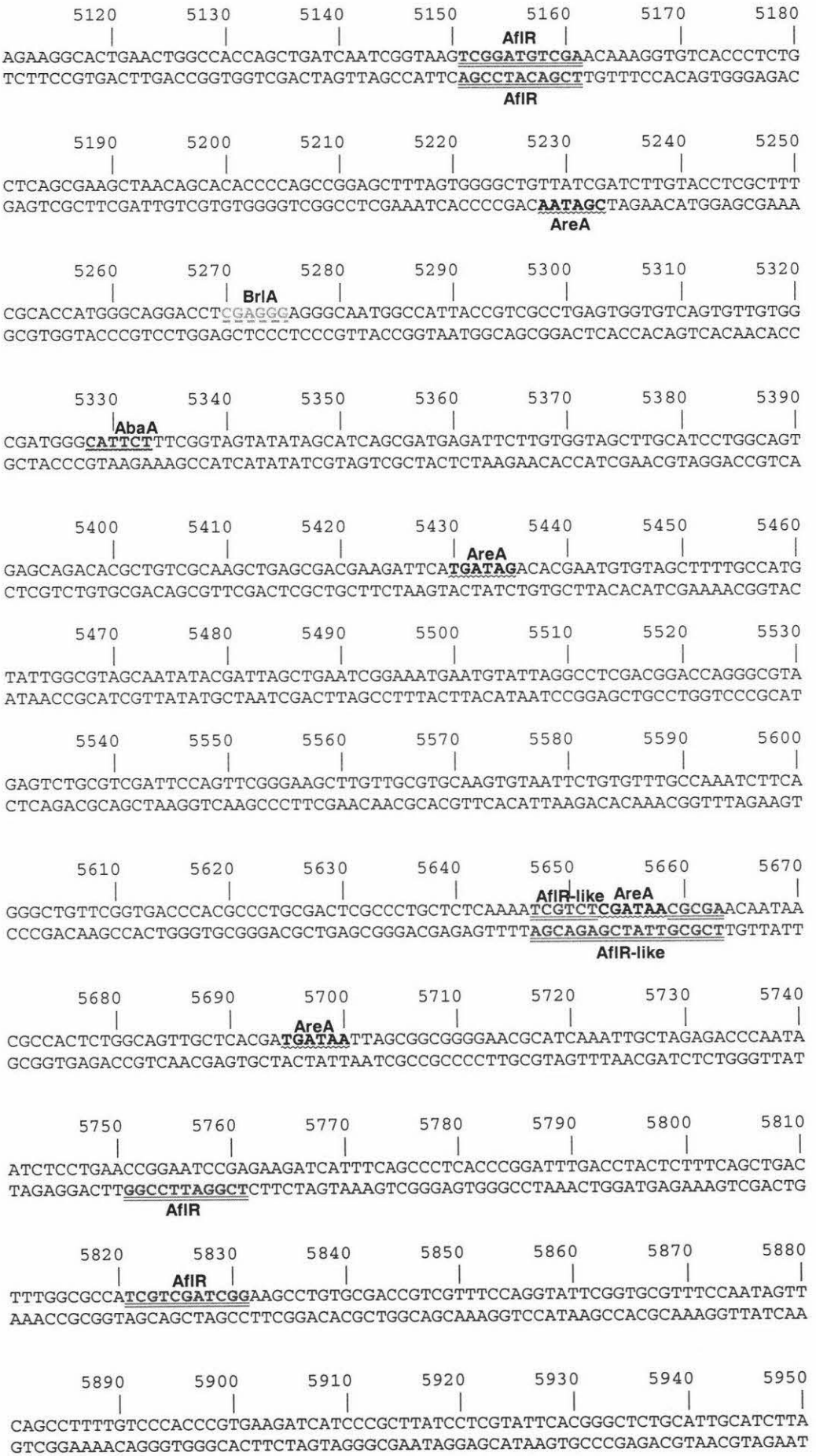
AreA binding motifs

BrlA binding motifs

PacC binding motifs

dotA and *dotB* intergenic region




5120 5130 5140 5150 5160 5170 5180
AflR
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TCTTCCGTGACTTGACCGGTGGTCGACTAGTTAGCCATTCAGCCTACAGCTTGTTTCCACAGTGGGAGAC
AflR
5190 5200 5210 5220 5230 5240 5250
CTCAGCGAAGCTAACAGCACACCCCAGCCGGAGCTTTAGTGGGGCTGTTATCGATCTTGTACCTCGCTTT
GAGTCGCTTCGATTGTCGTGTGGGGTCGGCCTCGAAATCACCCCGACAATAGCTAGAACATGGAGCGAAA
AreA
5260 5270 5280 5290 5300 5310 5320
BrlA
CGCACCATGGGCAGGACCTCGAGGGAGGGCAATGGCCATTACCGTCGCCTGAGTGGTGTCAGTGTTGTGG
GCGTGGTACCCGTCCTGGAGCTCCCTCCCGTTACCGGTAATGGCAGCGGACTCACCACAGTCACAACACC
5330 5340 5350 5360 5370 5380 5390
AbaA
CGATGGGCATTCTTTCGGTAGTATATAGCATCAGCGATGAGATTCTTGTGGTAGCTTGCATCCTGGCAGT
GCTACCCGTAAGAAAGCCATCATATATCGTAGTCGCTACTCTAAGAACACCATCGAACGTAGGACCGTCA
5400 5410 5420 5430 5440 5450 5460
AreA
GAGCAGACACGCTGTCGCAAGCTGAGCGACGAAGATTCATTGATAGACACGAATGTGTAGCTTTTGCCATG
CTCGTCTGTGCGACAGCGTTCGACTCGCTGCTTCTAAGTACTATCTGTGCTTACACATCGAAAACGGTAC
5470 5480 5490 5500 5510 5520 5530
TATTGGCGTAGCAATATACGATTAGCTGAATCGGAAATGAATGTATTAGGCCTCGACGGACCAGGGCGTA
ATAACCGCATCGTTATATGCTAATCGACTTAGCCTTTACTTACATAATCCGGAGCTGCCTGGTCCCGCAT
5540 5550 5560 5570 5580 5590 5600
GAGTCTGCGTCGATTCCAGTTCGGGAAGCTTGTTGCGTGCAAGTGTAATTCTGTGTTTGCCAAATCTTCA
CTCAGACGCAGCTAAGGTCAAGCCCTTCGAACAACGCACGTTCACATTAAGACACAAACGGTTTAGAAGT
5610 5620 5630 5640 5650 5660 5670
AflR-like AreA
GGGCTGTTCGGTGACCCACGCCCTGCGACTCGCCCTGCTCTCAAAATCGTCTCGATAACGCGAACAATAA
CCCGACAAGCCACTGGGTGCGGGACGCTGAGCGGGACGAGAGTTTTAGCAGAGCTATTGCGCTTGTTATT
AflR-like
5680 5690 5700 5710 5720 5730 5740
AreA
CGCCACTCTGGCAGTTGCTCACGATTGATAATTAGCGGCGGGGAACGCATCAAATTGCTAGAGACCCAATA
GCGGTGAGACCGTCAACGAGTGCTACTATTAATCGCCGCCCCTTGCGTAGTTTAACGATCTCTGGGTTAT
5750 5760 5770 5780 5790 5800 5810
ATCTCCTGAACCGGAATCCGAGAAGATCATTTCAGCCCTCACCCGGATTTGACCTACTCTTTCAGCTGAC
TAGAGGACTTGGCCTTAGGCTCTTCTAGTAAAGTCGGGAGTGGGCCTAAACTGGATGAGAAAGTCGACTG
AflR
5820 5830 5840 5850 5860 5870 5880
AflR
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AAACCGCGGTAGCAGCTAGCCTTCGGACACGCTGGCAGCAAAGGTCCATAAGCCACGCAAAGGTTATCAA
5890 5900 5910 5920 5930 5940 5950
CAGCCTTTTGTCCCACCCGTGAAGATCATCCCGCTTATCCTCGTATTCACGGGCTCTGCATTGCATCTTA
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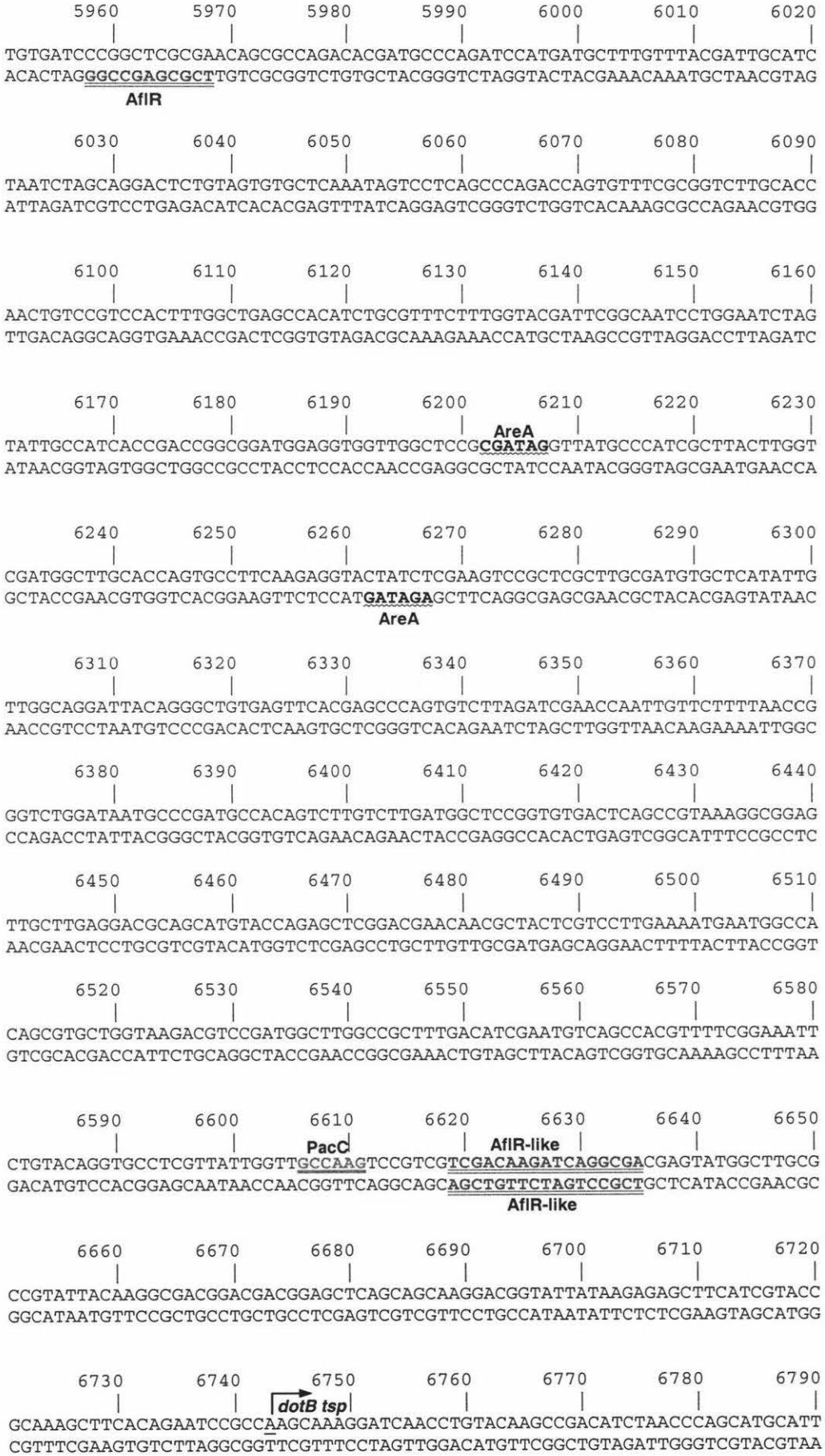
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AflR
6030 6040 6050 6060 6070 6080 6090
TAATCTAGCAGGACTCTGTAGTGTGCTCAAATAGTCCTCAGCCCAGACCAGTGTTTCGCGGTCTTGCACC
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6100 6110 6120 6130 6140 6150 6160
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TTGACAGGCAGGTGAAACCGACTCGGTGTAGACGCAAAGAAACCATGCTAAGCCGTTAGGACCTTAGATC
6170 6180 6190 6200 6210 6220 6230
AreA
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6240 6250 6260 6270 6280 6290 6300
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AreA
6310 6320 6330 6340 6350 6360 6370
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AACCGTCCTAATGTCCCGACACTCAAGTGCTCGGGTCACAGAATCTAGCTTGGTTAACAAGAAAATTGGC
6380 6390 6400 6410 6420 6430 6440
GGTCTGGATAATGCCCGATGCCACAGTCTTGTCTTGATGGCTCCGGTGTGACTCAGCCGTAAAGGCGGAG
CCAGACCTATTACGGGCTACGGTGTCAGAACAGAACTACCGAGGCCACACTGAGTCGGCATTTCCGCCTC
6450 6460 6470 6480 6490 6500 6510
TTGCTTGAGGACGCAGCATGTACCAGAGCTCGGACGAACAACGCTACTCGTCCTTGAAAATGAATGGCCA
AACGAACTCCTGCGTCGTACATGGTCTCGAGCCTGCTTGTTGCGATGAGCAGGAACTTTTACTTACCGGT
6520 6530 6540 6550 6560 6570 6580
CAGCGTGCTGGTAAGACGTCCGATGGCTTGGCCGCTTTGACATCGAATGTCAGCCACGTTTTCGGAAATT
GTCGCACGACCATTCTGCAGGCTACCGAACCGGCGAAACTGTAGCTTACAGTCGGTGCAAAAGCCTTTAA
6590 6600 6610 6620 6630 6640 6650
PacC
AflR-like
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GACATGTCCACGGAGCAATAACCAACGGTTCAGGCAGCAGCTGTTCTAGTCCGCTGCTCATACCGAACGC
AflR-like
6660 6670 6680 6690 6700 6710 6720
CCGTATTACAAGGCGACGGACGACGGAGCTCAGCAGCAAGGACGGTATTATAAGAGAGCTTCATCGTACC
GGCATAATGTTCCGCTGCCTGCTGCCTCGAGTCGTCGTTCCTGCCATAATATTCTCTCGAAGTAGCATGG
6730 6740 6750 6760 6770 6780 6790
dotB tsp
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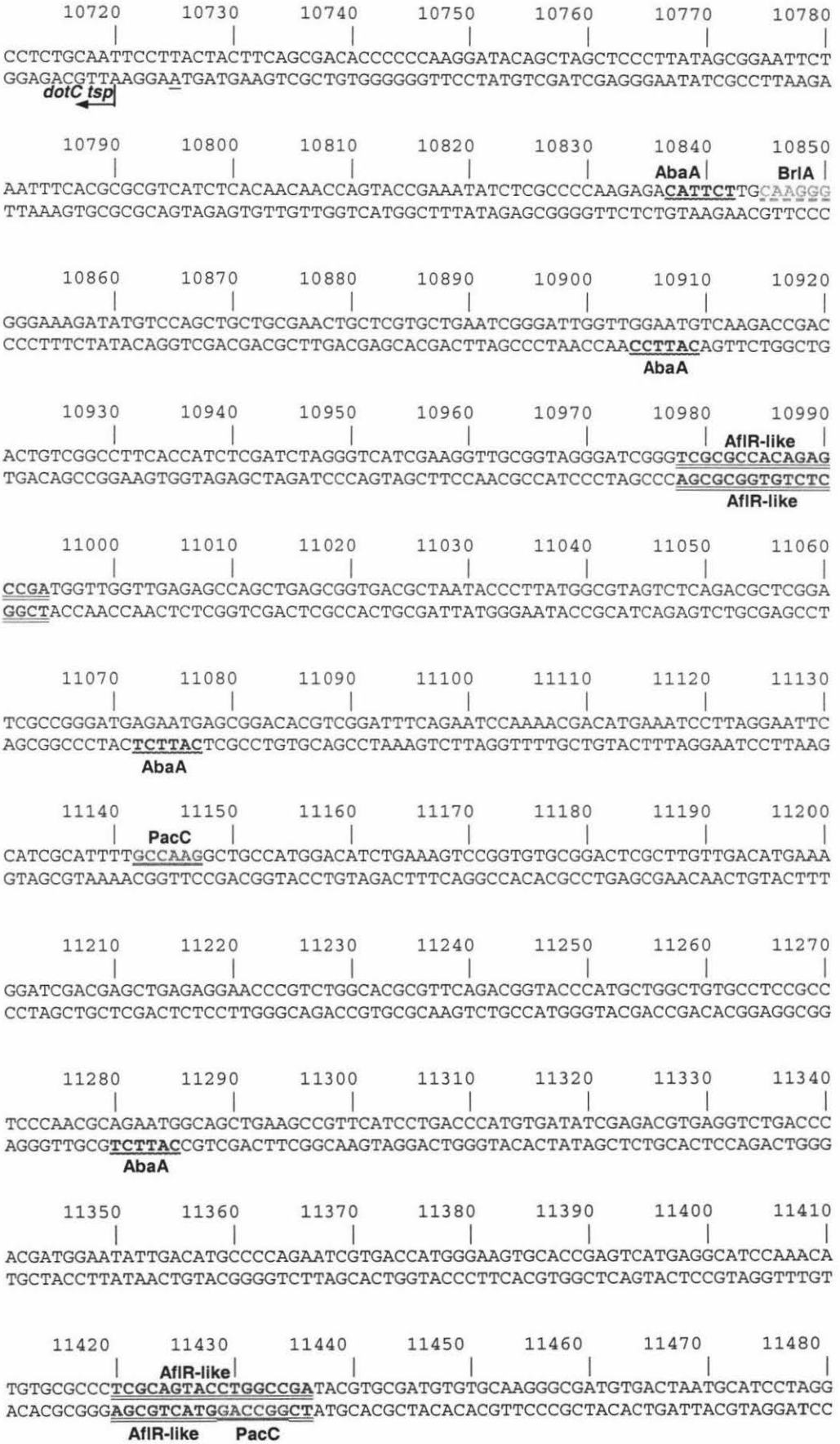
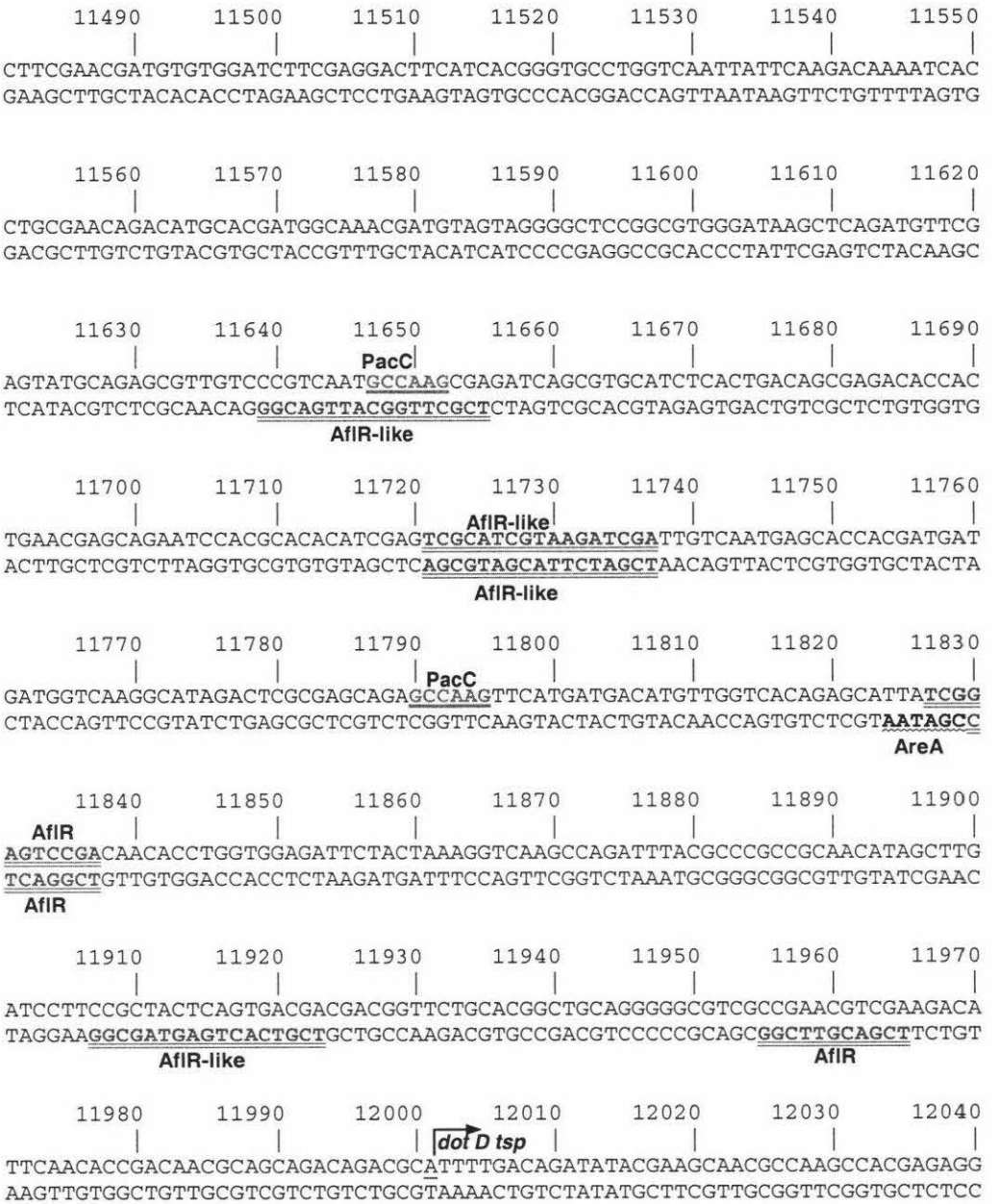
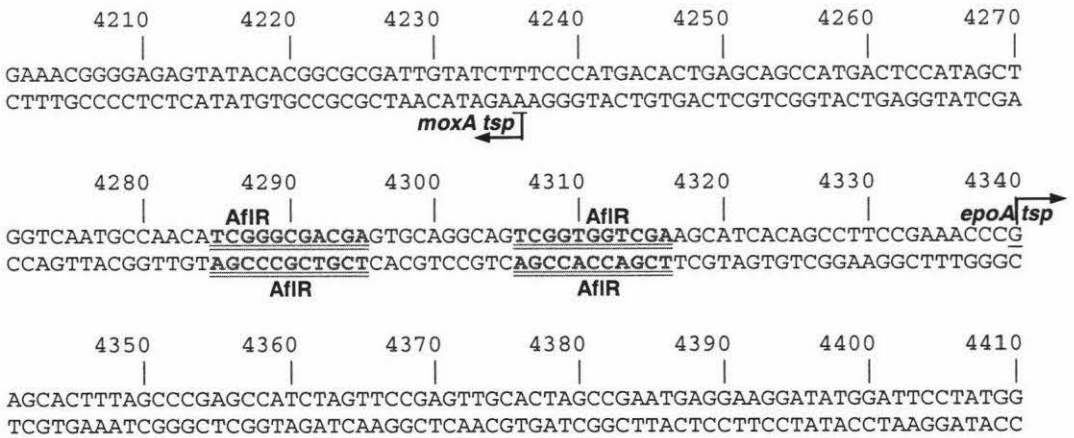
***dotC* and *dotD* intergenic region**

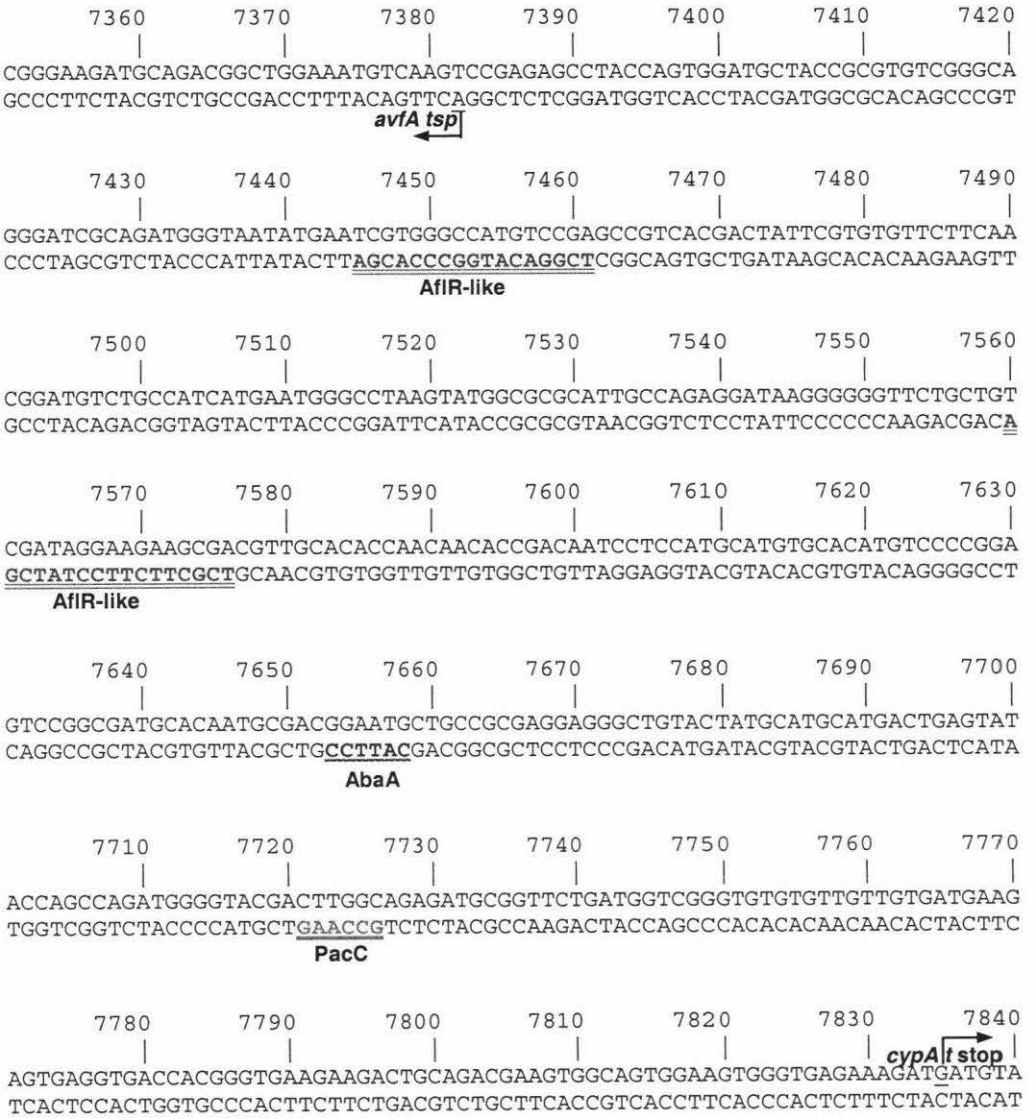




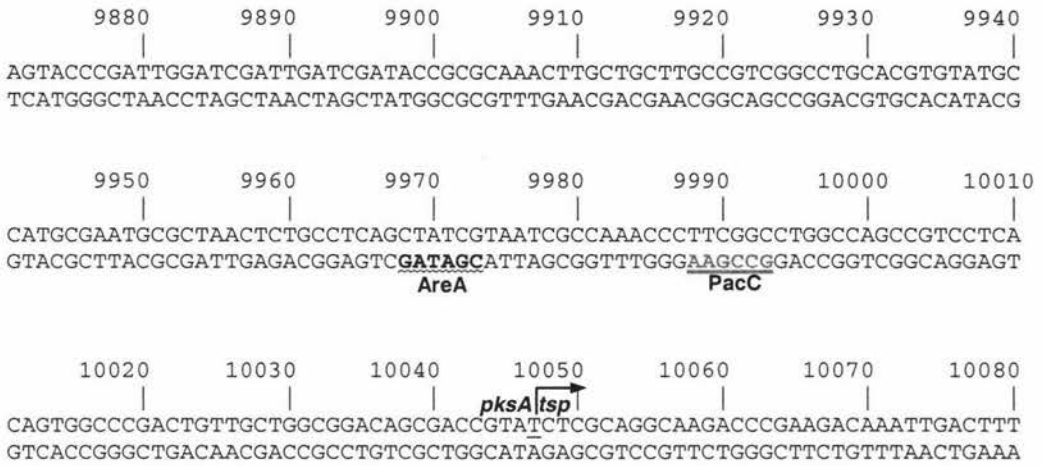
***moxA* and *epoA* intergenic region**



***avfA* and 3'*cypA* intergenic region**



***cypA* and *pksA* intergenic region**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9950 9960 9970 9980 9990 10000 10010
CATGCGAATGCGCTAACTCTGCCTCAGCTATCGTAATCGCCAAACCCTTCGGCCTGGCCAGCCGTCCTCA
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AreA
PacC
10020 10030 10040 10050 10060 10070 10080
pksA tsp
CAGTGGCCCGACTGTTGCTGGCGGACAGCGACCGTATCTCGCAGGCAAGACCCGAAGACAAATTGACTTT
GTCACCGGGCTGACAACGACCGCCTGTCGCTGGCATAGAGCGTCCGTTCTGGGCTTCTGTTTAACTGAAA