CONFIRMATION OF THE PRESENCE OF A DOTHISTROMIN BIOSYNTHETIC GENE CLUSTER IN THE FUNGAL FOREST PATHOGEN *DOTHISTROMA PINI.*

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ERRATUM SHEET

Figure 3.4 The term transformation event refers to each independent protoplast transformed with the disruption vector.

Figure 3.11 Dothistromin samples taken from colony 8A1 and wild type were diluted 10 fold. For qualitative comparison to undiluted dothistromin samples taken from *dotA*$^-$ colonies, % inhibition for wild type and colony 8A1 samples were multiplied by 10.

Appendix 1C and appendix 1D DNA obtained from *D. pini* and present in plasmids pR204 and pR208 is represented by

ABSTRACT

The polyketide dothistromin is a toxin produced by the fungus *Dothistroma pini* and is thought to play a role in causing *Dothistroma* needle blight on the pine *Pinus radiata.* Dothistromin is structurally similar to aflatoxin B1 (AF), a highly carcinogenic toxin with no known function, that is produced by the fungus *Aspergillus parasiticus.* The structural similarities between AF and dothistromin suggest that genes homologous to AF biosynthetic genes found in *D. pini* are dothistromin biosynthetic genes. AF biosynthetic genes in *A. parasiticus* and *A. flavus* are clustered, as are the biosynthetic genes of the structurally similar sterigmatocystin in *A. nidulans.* Dothistromin biosynthetic genes are also likely to be clustered. Two λ clones, λCGV1 and λBMKSA, containing different portions of this putative dothistromin cluster, have been isolated in previous studies.

In this study one gene contained on the clone λCGV1 coding for a putative dothistromin ketoreductase *dotA* (80.2% identical to *A. parasiticus* AF biosynthetic gene *ver-1*) was disrupted with the hygromycin B resistance gene (*hph)* using targeted disruption via homologous recombination. *dotA*$^-$ mutants were tested for dothistromin production and shown to produce at least 10 – 43 times less than the wild type strain. This confirmed that *dotA* is involved in dothistromin biosynthesis. Further more, *dotA*$^-$ mutants accumulated the intermediate versicolorin A. This finding provides evidence that λCGV1 contains a portion of the dothistromin biosynthetic gene cluster and the presence of versicolorin A suggests pathway by which dothistromin is synthesised. Other genes homologous to AF and ST biosynthetic genes contained on λCGV1 can now be disrupted in order to determine the extent of the dothistromin biosynthetic cluster on λCGV1. Dothistromin deficient mutants can also be used to determine the role of dothistromin in the pathogenicity of *D. pini.*

Further nucleotide sequencing of the clone λBMKSA revealed the promoter region and the N terminal amino acid encoding sequence of the putative dothistromin polyketide synthase PKS^{DOT}. The partial PKS^{DOT} sequence (amino acids 1-1426) is 62% identical to the *A. parasiticus* PKSA involved in AF biosynthesis. Preparations to disrupt PKS^{DOT} were made and disruption will confirm its presence on the dothistromin biosynthetic pathway.

Sequencing of λBMKSA in this study also revealed a putative dothistromin p450 mono-oxygenase gene, *dcm1,* providing more evidence that λBMKSA contains part of the dothistromin pathway. The amino acid sequence of *dcm1* is 59% identical to CYPX from the *A. parasiticus* AF cluster and 56% identical to STCB from the *A. nidulans* ST cluster. The function of these homologs has not been ascertained. The discovery of a homolog in *D. pini* (a species only thought to contain genes for the first part of the AF/ST pathway) provides information about the function of these homologs in AF and ST biosynthesis.

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1.0 INTRODUCTION

New Zealand has a rapidly growing forestry industry currently contributing $2.5 billion in exports (3.9% of New Zealand's national income), making it New Zealand's third largest export industry with a huge potential for expansion.

Radiata Pine (*Pinus radiata)* makes up 90% of the 1.7 million hectares of New Zealand's planted forest. The area of planted forest in New Zealand is rapidly expanding. The introduction and establishment of an exotic pest could result in serious depletion of New Zealand's pine forests and with it, economic devaluation. The forest pathogen *Dothistroma pini* is an exotic fungus that has been introduced and causes *Dothistroma* needle blight.

1.1 *DOTHISTROMA* NEEDLE BLIGHT IN *PINUS RADIATA.*

Dothistroma needle blight in *Pinus radiata* is a result of infection by the fungus *Dothistroma pini*. *D. pini* Hulbary was first noted in New Zealand in 1962 and is known to cause premature defoliation and on occasion death of trees. The fungus causes the appearance of characteristic red bands on the needles of the pine, with black fruiting bodies also able to be seen.

Aside from premature defoliation of the infected *P. radiata* needles, the major consequence of *D. pini* infection is the loss of volume gain (Gadgil 1984). The loss of volume gain of *P. radiata* due to *D. pini* infection is estimated to cost the New Zealand forestry industry $6.67 million per year (Carson and Carson 1991). Most of the North island of New Zealand is infected with *D. pini* (excluding the northern tip) as well as Nelson, parts of Marlborough, Westland, Southland and Otago in the South Island. Outbreaks have also been reported in many other areas of the world (Gadgil 1984).

Dothistroma needle blight is thought to be correlated to the secretion of the toxin dothistromin by the fungus. Injection of pure dothistromin into pine needles has been

observed to reproduce symptoms of *Dothistroma* needle blight. From these observations it was concluded that dothistromin was a phytotoxin and responsible for the needle blight, however its exact role in the disease is yet to be ascertained (Stoessl, Abramowski *et al.* 1990). To determine that dothistromin does play a role in *Dothistroma* needle blight, dothistromin deficient mutants need to be made (This study) and the pathogenicity of these mutants tested.

1.2 INFECTION

D. pini infects its host when the fungal hyphae contact the stoma of the plant and enter with appressorium like structures (Franich 1983). However, the infection relies on favourable environmental conditions such as low light (Gadgil and Holden 1976). The development of stromata (fruiting bodies) of *D. pini* is inhibited by the absence of leaf surface moisture, therefore continuous moisture increases infection (Gadgil 1977). The age of *P. radiata* trees also affects susceptibility to infection; younger trees are more susceptible as their stomata are open pores and allow easier access for the appressorium of the fungus (Gadgil 1967)

1.3 CURRENT INFECTION CONTROL METHODS

As *Dothistroma* needle blight is a risk to New Zealand's forestry industry, efficient and economic control methods need to be used to reduce the incidence of *Dothistroma* needle blight. Two methods currently being employed to reduce the blight are detailed below.

1.3.1 Chemical control

Dothistroma needle blight can be controlled by spraying copper-based fungicides. Studies in New Zealand proved aerial spraying by copper oxychloride was feasible as a control method (Dick 1989). Copper spraying exceeding 100,000 ha/annum has been carried out in New Zealand since the 1970's in areas where annual surveys show in excess of 25% of crown infection by *D. pini*. Aerial spraying has been found to reduce, but not eliminate, the

disease bringing into question the economic benefits of such a process (Carson, Dick *et al.* 1991).

1.3.2 Resistant strains

In stands of infected trees, it was observed that some trees were resistant to infection by *D. pini*. Resistance of *P. radiata* to *D. pini* infection was shown to be a heritable trait and a resistant cultivar was developed by selective breeding of resistant *P. radiata* strains (Carson 1989). In most programs disease resistance is bred for as well as non-disease linked traits such as growth and form. In New Zealand this has been implemented by using trees already improved for other traits to breed for *D. pini* resistance (Carson and Carson 1989).

Using the *Dothistroma* resistant breed of *P. radiata* will reduce mean stand infection by 15% and result in a 56% reduction in spraying costs as well as recovery of growth loss due to *D. pini* infection (Carson, Dick *et al.* 1991). It is suggested by the NZ Forest Research Institute that the *Dothistroma* resistant breed is used only in areas at risk of *D. pini* infection and normal sites have the standard breeds planted. This is suggested as the resistant breed has a lower yield on normal sites (Dick 1989). Spraying of copper fungicides as well as resistance will give additive effects against *D. pini* (Carson, Dick *et al.* 1991).

1.4 THE BIOCHEMISTRY AND MOLECULAR BIOLOGY OF DOTHISTROMIN BIOSYNTHESIS.

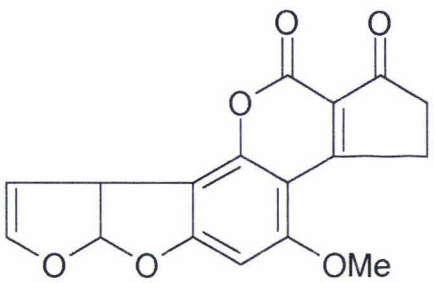
1.4.1 Dothistromin Biochemistry

Dothistromin is a fungal metabolite that has been isolated from *D. pini*, several *Cercospora* sp. and *Mycosphaerella laricina*. Dothistromin has a similar difuran structure to versicolorin B, an intermediate in the aflatoxin (AF) pathway of *Aspergillus parasiticus* (Figure 1.1). These both belong to a group of compounds all of which have a

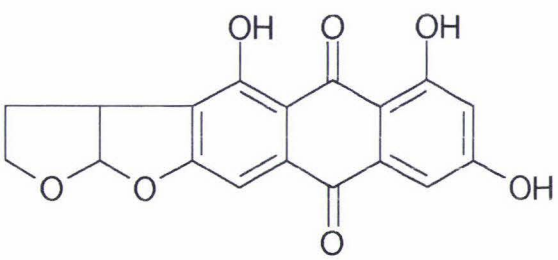
furobenzofuran moiety (Bassett and Buchanan 1970). Others in the group are AF and sterigmatocystin (ST) (Gallagher and Hodges 1972). The tetrahydro-2-hydroxy-bisfuran moiety that is found in dothistromin is also found in aflatoxin B1 (AFB_1) where it is thought to confer hepatotoxic and carcinogenic characteristics. Following these observations dothistromin was tested, found to be mutagenic and have potential carcinogenic qualities (Elliott, Mason *et al.* 1989).

Six anthraquinones apart from dothistromin have been found in *D. pini*. The structures of these have an organization of anthraquinone fused to dihydrofuran rings. Three of the compounds that have rings fused in this way (averantin, averufin and versicolorin B) (figure 1.2) are present in *Aspergillus* species as intermediates on AF and ST pathways (Danks and Hodges 1974). These similarities suggest that the fusion of the rings in the dothistromin intermediates could occur in the same way as AF intermediates. Structural similarity of dothistromin to AF and ST also suggests these compounds could have common intermediates (Shaw, Chick *et al.* 1978).

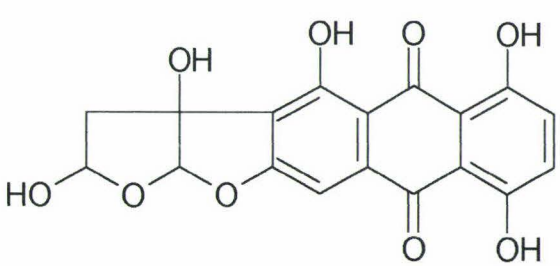
Figure 1.1 Comparison of aflatoxin, sterigmatocystin intermediate versicolorin B and dothistromin.



aflatoxin B1



versicolorin B



dothistromin

1.4.2 AFLATOXIN

Aflatoxins are secondary metabolites produced by *A. parasiticus*, *A. flavus* and *A. nomius*. These fungi are widespread and when they infect stored grains and nuts, cause contamination of the food with carcinogenic aflatoxins.

The pathway of aflatoxin B1 (AFB1) is well established due to classical experiments with AFB1 deficient mutants reviewed in (Minto and Townsend 1997). Genes on this pathway such as the gene responsible for the conversion of the intermediate versicolorin A into ST have been cloned, isolated and sequenced (figure 1.2).

An AF deficient mutant of *A. parasiticus* produced yellow pigments. The major yellow pigment was identified as versicolorin A. As versicolorin A has the difuran group unique to AF it was proposed to be an intermediate of the AF pathway in *A. parasiticus* (Lee, Bennett *et al.* 1975). A cosmid genomic DNA library of wild type *A. parasiticus* was made and was transformed into $AFB1^-$ mutants lacking in the conversion of the intermediate versicolorin A into ST. An $AFB1^+$ transformant was found and the gene on the cosmid responsible for the complementation was named *ver-1*.

The amino acid sequence predicted for *ver-1* had similarities to ketoreductases involved in polyketide biosynthesis in *Streptomyces*. Nucleotide sequence analysis established that the *ver-1* gene encodes a protein involved in the modification of ring structures like that of the conversion of versicolorin A to ST (Skory, Chang *et al.* 1992). A similar gene called *stcU* was found in the ST biosynthetic pathway of *A. nidulans*. This was also thought to be involved in the versicolorin A to ST step (Keller, Kantz *et al.* 1994). What was thought to be a duplicated copy of the *ver-1* gene was found in *A. parasiticus* and called *ver-1B*. The nucleotide sequence of *ver-1B* was deduced and was found to be a truncated gene with a non-functional translation product. The *ver-1B* amino acid sequence was found to have 95% identity to the *ver-1* sequence (Liang, Skory *et al.* 1996).

The genes responsible for the biosynthesis of AF and ST are clustered within the genome. Gene clusters are two or more genes that are closely linked and participating in the same metabolic or developmental pathway. Examples have been long acknowledged in bacterial genomes but only recently been recognised in eukaryotic genomes. Two genes, *ver-1* and *nor-1* in *A. parasiticus* were localised and used to determine whether other genes in this area were involved in AF synthesis. 14 RNA transcripts from *A. parasiticus* grown in AF inducing medium were localised to this region. Several genes were physically linked on a cosmid and chromosome. This and findings in *A. nidulans* suggest that genes involved in AF biosynthesis are clustered in the genome of *Aspergillus* species (Trail, Mahanti *et al.* 1995). Nine of the genes involved in the AF pathway were found on the same 60-kb fragment (Yu and Leonard 1995). 19 putative AF genes have now been found on this 60 kb fragment (Yu, Chang *et al.* 2000) (figure 1.2). A ST synthesis cluster in *A. nidulans* has been sequenced and twenty-five genes thought to be involved in ST synthesis have been identified. The ST cluster has a different order of genes to the AF cluster, but the gene products are very similar between the two. Other genes unlinked to the AF/ST clusters have been identified that are also thought to be involved in synthesis of AF and ST (Woloshuk, Foutz *et al.* 1994).

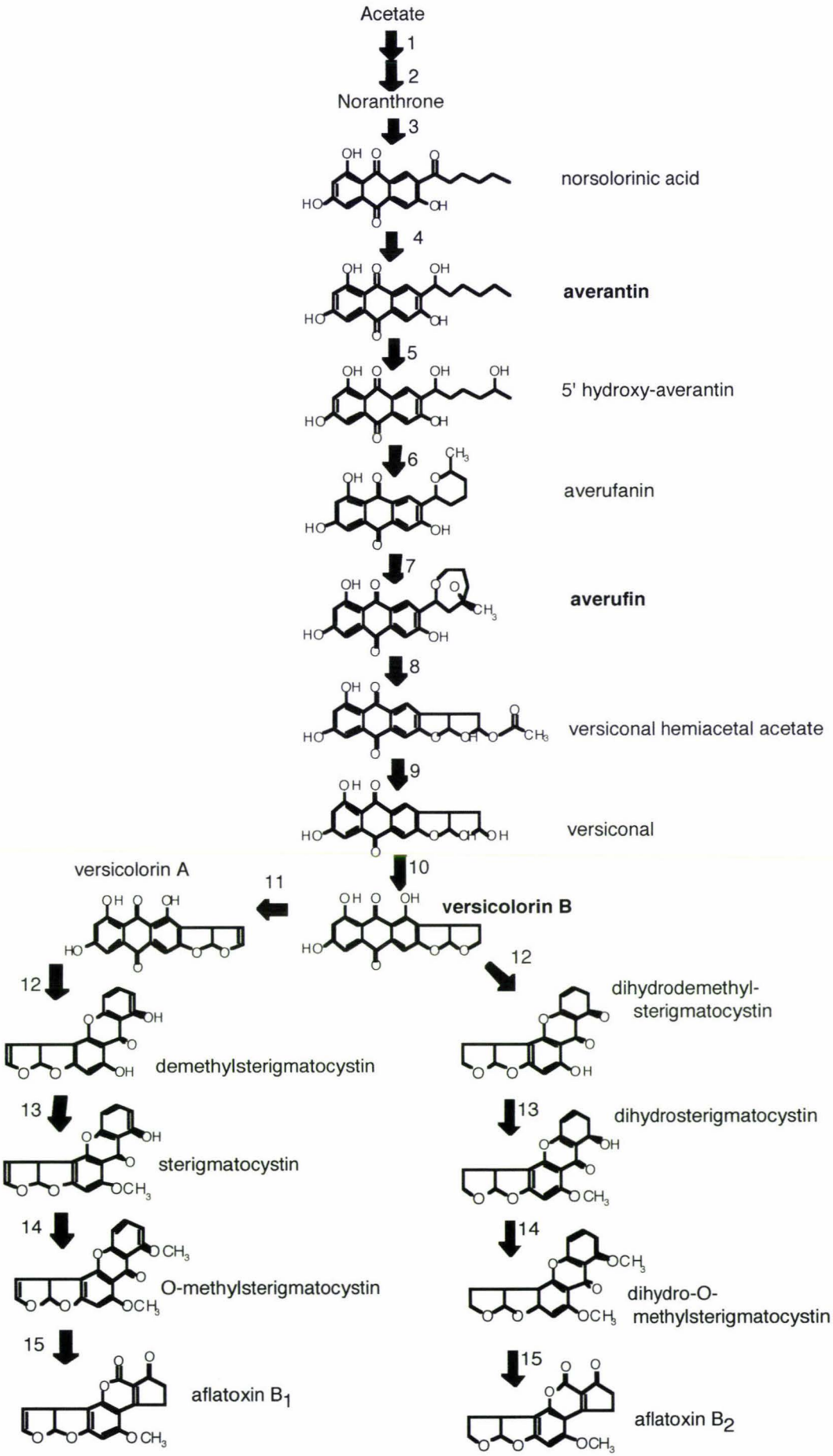
1.4.3 DOTHISTROMIN MOLECULAR BIOLOGY

The structural and chemical similarities of dothistromin to the mycotoxins ST and AF produced by *Aspergillus* species and the sequences of the genes involved in these pathways that have been cloned, have been used to isolate and characterise putative genes on the dothistromin biosynthetic pathway. Similarities between AF and ST pathways with the dothistromin pathway have allowed gene sequences known to be on AF/ST pathways to be used as hybridisation probes for the isolation of dothistromin genes. Probing a *D. pini* genomic library with the *ver-1* gene from *A. parasiticus* resulted in the isolation of two clones, λCGV1 and λCGV2 (Gillman 1996).

Figure 1.2 ***A. parasiticus*** **aflatoxin biosynthetic pathway cluster with comparison to biochemical pathway.**

The figure shows the action of genes on the aflatoxin biosynthetic pathway in *A. parasiticus and A. flavus*. The genes are listed below with their function. The number corresponds to the pathway step they catalyse. Intermediates in bold are also found in *D. pini*. The diagram is based on a similar one by Brown *et al*, 1999. Genes are listed in order as they appear in the *A. parasiticus* AF gene cluster.

Gene	Product Function	Pathway step
norB	Dehydrogenase	4
pksA	Polyketide synthase	2
nor-1	Reductase	4
fas-2	Fatty acid synthase-2	1
fas-1	Fatty acid synthase-1	1
aflR	Pathway regulator	
adhA	Alcohol dehydrogenase	6 or 7
estA	Esterase	9
norA	Aryl-alcohol dehydrogenase	4
ver-1	Dehydrogenase	12
verA	Monooxygenase	12
avnA	P450 Monooxygenase	5
verB	Desaturase	11
avfA	Oxidase	8
omtB	Methyltransferase B	13
omtA	Methyltransferase A	14
ordA	Oxidoreductase (p450 enzyme)	15
vbs	Ver B Synthase	10
cypX	P450 Monooxygenase	*unconfirmed*
moxY	Monooxygenase	*unconfirmed*


Acetate
1
2
Noranthrone
3
norsolorinic acid
4
averantin
5
5' hydroxy-averantin
6
averufanin
7
averufin
8
versiconal hemiacetal acetate
9
versiconal
10
versicolorin B
11
versicolorin A
12
demethylsterigmatocystin
13
sterigmatocystin
14
O-methylsterigmatocystin
15
aflatoxin B1
12
dihydrodemethyl-
sterigmatocystin
13
dihydrosterigmatocystin
14
dihydro-O-
methylsterigmatocystin
15
aflatoxin B2

Sequencing of λCGV1 revealed 5 genes within a 13.3 kb region (Monahan 1998). These genes were named *dotA* (dothistromin ketoreductase), *dotB* (dothistromin oxidase), *dotC* (dothistromin toxin pump), *ddh1* (dothistromin dehydrogenase 1) and *dotD* (dothistromin thioesterase). Their functions were postulated based on amino acid sequence homology to genes in the AF gene cluster in *A. parasiticus* (figure 1.3 & table 1.1).

Figure 1.3 Comparison between *A. parasiticus* AF gene cluster and *A. nidulans* ST gene cluster.

The boxes represent the position of genes within each cluster and arrows represent the direction of transcription. The coloured boxes contain the name of the gene. The names of homologs in each cluster are contained in the same coloured boxes. Grey boxes indicate homologs are unknown. Based on a diagram by D. Bhatnagar (Personal communication).

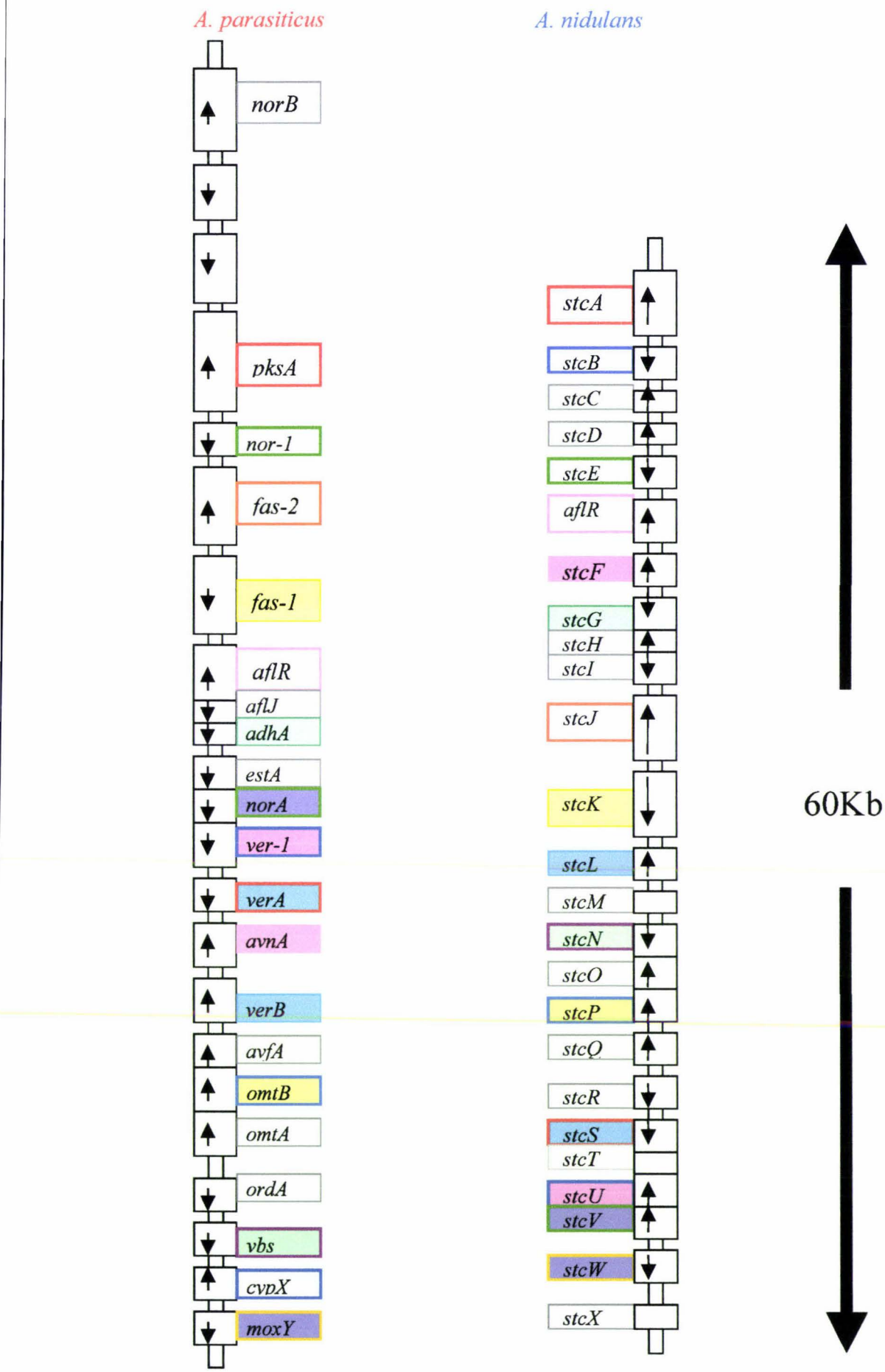

A. parasiticus
A. nidulans
norB
pksA
nor-1
fas-2
fas-1
aflR
aflJ
adhA
estA
norA
ver-1
verA
avnA
verB
avfA
omtB
omtA
ordA
vbs
cypX
moxY
stcA
stcB
stcC
stcD
stcE
aflR
stcF
stcG
stcH
stcI
stcJ
stcK
stcL
stcM
stcN
stcO
stcP
stcQ
stcR
stcS
stcT
stcU
stcV
stcW
stcX
60Kb

Table 1.1 Homologs of putative dothistromin biosynthetic genes.

D. pini gene	Reference	Homologs	% identity (% similarity)	Organism	Function	Reference
dotA	Monahan (1998)	*ver-1*	80 (87)	*Aspergillus parasiticus*	Ketoreductase- AF biosynthesis	(Skory, Chang et al. 1992)
		stcU	79 (88)	*A. nidulans*	Ketoreductase -ST biosynthesis	(Keller, Kantz et al. 1994): (Brown, Yu et al. 1996)
		BRM2	65	*Alternaria alternata*		
		brn1	62 (76)	*Cochliobolus heterostrophus*	Reductase- melanin biosynthesis	
		thr1	59 (73)	*Colletotrichum lagenarium*	Reductase- melanin biosynthesis	(Shimizu, Takana et al. 1997)
		thnR	58 (72)	*Magnaporthe grisea*	Reductase- melanin biosynthesis	(Perpetua, Kubo et al. 1996)
						Vidal-Cros *et al.*, 1994
dotB	Monahan (1998)	*stcC*	24 (29)	*A. nidulans*	Oxidase-ST biosynthesis	(Brown, Yu et al. 1996)
		Chloro-peroxidase gene	24 (31)	*Caldariomyces fumago*	Chloroperoxidase- biosynthesis of chlorinated secondary metabolites.	(Nuell, Fang et al. 1988)
dotC	Monahan (1998)	*aflT*	31	*A. parasiticus*	Putative AF pump.	Unpublished
		ToxA	31 (41)	*C. carbonum*	HC-toxin efflux pump	(Pitkin 1996)
dotD	Monahan (1998)	TE[1] from :				
		stcA	37 (38) [2]	*A. nidulans*	PKS –ST biosynthesis	(Yu and Leonard 1995)
		pksL1	35 (45)	*A, parasiticus*	PKS – AF biosynthesis	(Feng and Leonard 1995)
		pksP	39 (58)	*A. fumigatus*	PKS – conidial pigmentation Biosynthesis	
		pks1	35 (42)	*C. lagenarium*	PKS – Melanin biosynthesis	(Takano, Kubo et al. 1995)

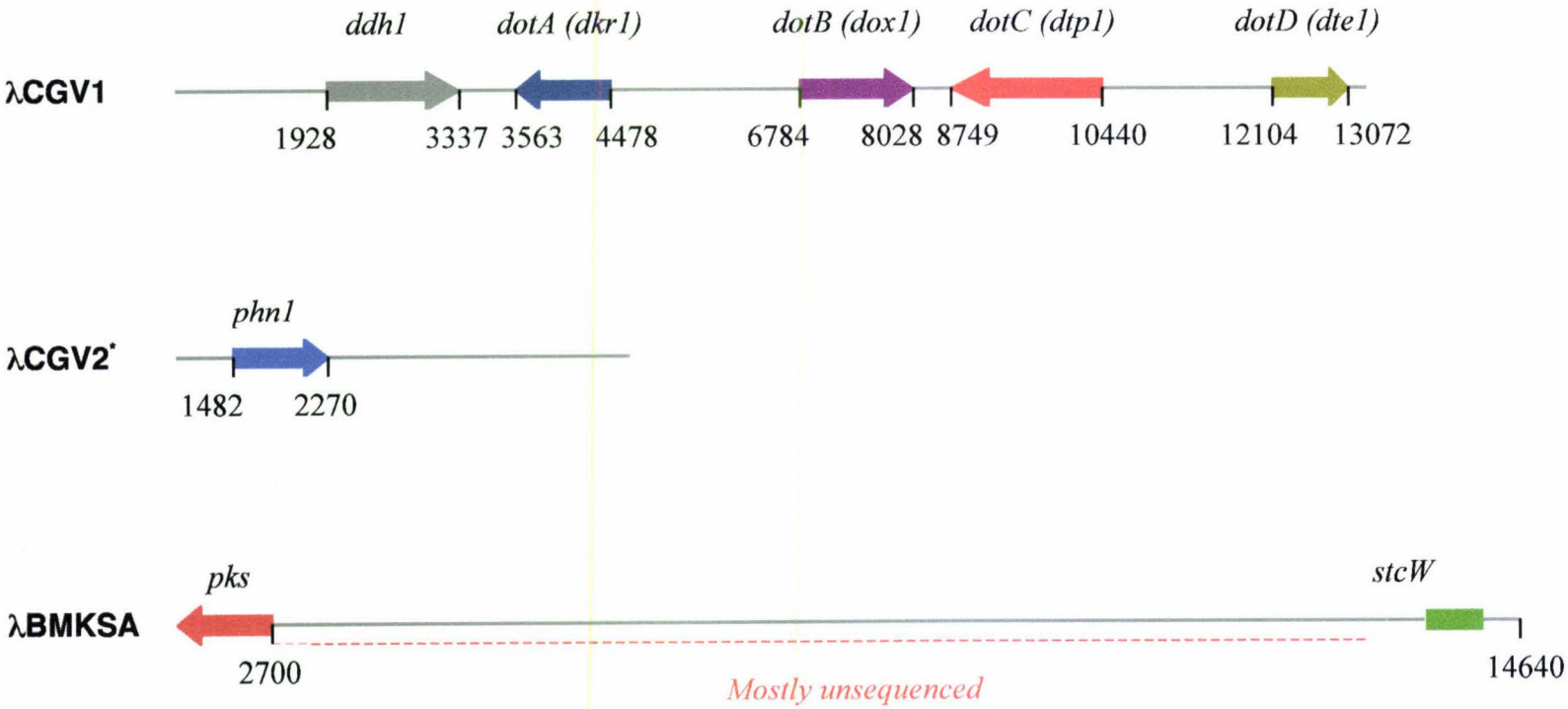
[1] Thioesterase domain

[2] Identities and similarities to TE domains of the gene only.

Figure 1.4 *D. pini* λ clones showing putative dothistromin biosynthetic genes.

A schematic, diagram (not to scale) showing *D. pini* λ clones that contain open reading frames thought to be involved in dothistromin biosynthesis. Coloured arrows indicate putative dothistromin biosynthetic genes. Gene names in brackets are names used in previous publications. The gene *ddh1* is not thought to be involved in the dothistromin biosynthetic pathway.

The orientation of clones relative to each other within the genome is unknown at this time.


ddh1
dotA (dkr1)
dotB (dox1)
dotC (dtp1)
dotD (dte1)
λCGV1
1928
3337
3563
4478
6784
8028
8749
10440
12104
13072
phn1
λCGV2*
1482
2270
pks
stcW
λBMKSA
2700
14640
Mostly unsequenced

As the AF biosynthetic pathway and the dothistromin biosynthetic pathway are biochemically similar, the similarities with the *D. pini* genes and the AF genes allow these *D. pini* genes to be putatively called the dothistromin biosynthetic genes (Table 1.1). To confirm the gene clustering, Southern blot analysis of *D. pini* genomic DNA restriction fragments, showed that probes for each gene hybridised to the same large *Xba*I fragment (Monahan 1998).

The amino acid sequence of *dotA* has similarity to reductase and dehydrogenase genes. It bears particular resemblance to ketoreductases in ST and AF biosynthesis. Genes *dotA* is homologous to are the *A. parasiticus ver-1* gene with 80% identity (87% similarity), the *A. nidulans stcU* gene 79% (88%), the *Cochliobolus heterostrophus brn1* gene 64% (76%), the *Magneporthe grisea* 1,3,6,8-tetrahydroxynapthalene (T_4HN) reductase gene 58% (72%) and the *Colletotrichum lagenarium thr1* gene 59% (73%) (Monahan 1998). The *A. parasitcus ver-1* gene and *A. nidulans stcU* gene both code for ketoreductases that are thought to be at equivalent steps of ST/AF pathways. The similarities with AF and ST ketoreductases indicate that the *dotA* gene could catalyse the same step in dothistromin production. In order to conclusively show that the putative dothistromin biosynthetic genes are involved in dothistromin biosynthesis, targeted disruption of the genes must take place and the resulting mutants assayed for dothistromin production. Accumulation of intermediate metabolites in the mutant strains will indicate which step of the pathway *dotA* the gene acts at. This study will investigate if *dotA* from λCGV1 is essential for dothistromin biosynthesis in *D. pini*.

The presence of genes homologous to AF pathway genes in *D. pini* could be involved in ST/AF biosynthesis but *D. pini* does not produce AF under the conditions tested (Ganley, 2000).

phn1 (polyhydroxynapthalene reductase), another *ver-1* homologue in the *D. pini* λCGV2 clone, has been partially sequenced. The predicted amino acid sequence of *phn1* 75% identity and 87% similarity to the trihydroxynapthalene (T_4HN) reductase gene on the melanin biosynthetic pathway in the phytopathogenic fungus *M. grisea* (Laarakkers 1999) Trihydroxynapthalene (T_4HN) reductase is involved in the reduction of 1,3,8-trihydroxynapthalene (1,3,8-THN) to vermelone on the melanin biosynthetic pathway and *phn1* might to catalyse a similar step in *D. pini*.

1.4.4 Polyketide biosynthesis

Dothistromin, aflatoxin and sterigmatocystin, along with intermediates on their biosynthetic pathways, are polyketides. Polyketides are a group of structurally diverse compounds produced by both prokaryotes and eukaryotes for many varied biological roles, linked by the biosynthesis of their carbon backbone. All are derived from carbon chains made by successive condensation of simple carboxylic acid groups in a fashion similar to that of fatty acid synthesis (Reviewed in (Hopwood 1990)). The β-carbon in these groups always carries a keto group, which often isn't reduced. It is these non-reduced keto groups that give the group the name polyketides.

The diversity of the molecules within the group is due to the fate of the keto groups from the carboxylic acids added each round of condensation, the side chain variation of the second carbon group added from the carboxylic acid, the length of the carbon backbone (related to the number of rounds of condensation) and the chirality around the carbon atoms. The variations at the early stages of polyketide synthesis are controlled by the enzyme polyketide synthase (PKS), specific to each pathway.

1.4.4.1 Fungal Polyketide Synthases.

Fungal PKS enzymes are type I PKSs. That is, they are large multifunctional polypeptides encoding multiple subunits, each with a catalytic function and high sequence similarity to fatty acid synthases. The order of these catalytic subunits, determines the biochemistry of the resulting polyketide. A minimal fungal PKS contains an acyl transferase (AT) that selects the appropriate extender unit, a β keto-acyl synthase domain (KS) that catalyses the condensation reaction, and acyl-carrier protein (ACP) domain that holds onto the growing polyketide chain and takes the next extender unit from the AT domain ready for the next condensation reaction. These core domains produce a keto ester which can then be processed by optional reductive domains such as enoyl reductase (ER) ketoreductase (KR) and dehydratase (DH). If a reductive domain is not present, the keto group will remain unchanged. Each domain has a conserved sequence and domains share particularly high sequence similarity between organisms.

PKS genes have been cloned and characterised from several filamentous fungi including *A. nidulans* ((Yu and Leonard 1995)& (Mayorga and Timberlake 1992)), *A. parasiticus* (Chang, Cary *et al.* 1995), *Cochliobolus heterostrophus* (Yang, Turgeon *et al.* 1994)and *Colletotrichum lagenarium* (Takano, Kubo *et al.* 1995). For the arrangement of the domains within each polypeptide, see table 1.2. Duplicated ACP domains are a feature of several of the PKS enzymes shown in Table 1.2 but are not common. The significance of having tandem ACP domains is not yet clear especially when other PKS proteins in very similar pathways (such as the *A. parasiticus* PKSA involved in the aflatoxin biosynthetic pathway) have only one ACP domain. Not all PKS genes have a TE domain in the same ORF, although, this domain is present in both PKS^{ST} and PKSA. It has been suggested that the TE domain releases the product from the complex (Tai, Chirala *et al.* 1993). The PKS1 from *C. heterostrophus* contains all the domains for processing of the β-carbons except the TE domain.

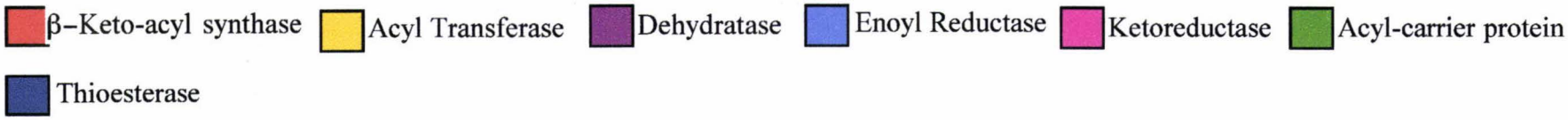
1.4.4.2 A *Dothistroma pini* Polyketide Synthase

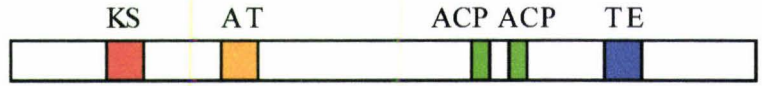
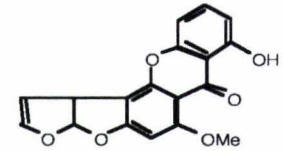
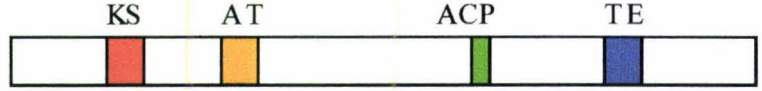
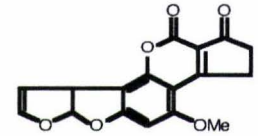
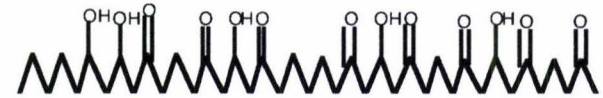
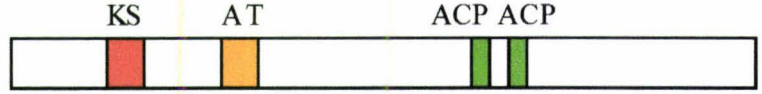
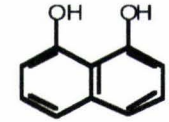
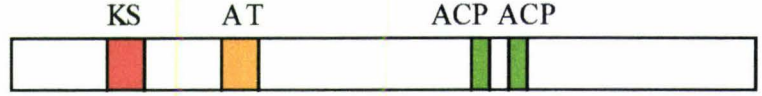
The similarity of the polyketides sterigmatocystin, aflatoxin and dothistromin has already been illustrated (figure 1.1). As described earlier, the biosynthetic pathways of these polyketides are thought to be very similar. Intermediates known to be on each of the pathways, have been found in sterigmatocystin, aflatoxin or dothistromin producing organisms suggesting some of the pathway components are the same. The initial reactions including the PKS catalysed step, are thought to be identical in all three pathways producing norsolorinic acid, despite the differences evident in the PKS genes cloned from *A. nidulans* and *A. parasiticus* for these reactions (Figure 1.5). It will be interesting to investigate the structure of *D. pini* PKS (PKS^{Dot}) for comparison of PKSs that can catalyse this same step.

The highly conserved sequence motifs present in the domains are able to be used as hybridisation probes for genomes thought to contain a homologous gene. A *D. pini* genomic library was probed with the β keto-acyl synthase domain from the *pksA* gene from *A. parasiticus* and hybridising clones were isolated (Morgan 1997). Sequence analysis of 2.4 Kb of the clone λBMKSA identified a portion of the *D. pini* PKS gene, two PKS domains: AT and KS. The sequence also revealed high amino acid identity to *A. parasiticus* PKSA and *A. nidulans* WA. From these results it has been postulated that the clone identified by the KS probe is part of PKS^{Dot} that is involved at the beginning of the dothistromin biosynthetic pathway. The *dotD* gene on λCGV1 (Monahan 1998)was sequenced and found to be homologous to TE domains in fungal PKS genes (Table 1.1). This suggests that, like in *C. heterostrophus*, the TE domain of PKS^{Dot} maybe on a separate polypeptide to the rest of the PKS.

Figure 1.5 Comparison Of PKSs In Different Species Of Fungi.

Figure shows the arrangement of conserved domains within single PKS polypeptide and the variety of final products resulting from the pathways the PKS acts on.



Organism	Gene	Protein structure	Product of Biosynthetic Pathway[1]
A. nidulans	*pks*ST (Yu & Leonard, 1995)		 Sterigmatocystiin
A. parasiticus	*pksL1* (Chang *et al*, 1995)		 Aflatoxin
C. heterostrophus	*pks1* (Yang *et al*, 1994)		 T-toxin
C. lagenarium	*pks1* (Takano *et al*, 1995)		 1,8-dihydroxynapthalene
A. nidulans	*wA* (Mayorga and Timberlake 1992)		Unknown spore pigment

[1] This table shows the final product of the biosynthetic pathway these PKSs are on, not the intermediate immediately following the PKS catalysed reaction.

1.5 TARGETED GENE DISRUPTION OF FUNGAL BIOSYNTHETIC GENES.

Targeted gene disruption in filamentous fungi has been demonstrated in the last decade by a number of groups. Gene targeting involves replacing or disrupting a wild type gene in the organism with another gene (often one that allows for positive selection of transformants). This disruption or replacement stops the wild type gene from functioning.

Gene disruption has been demonstrated in many filamentous fungi such as *Alternaria alternata* (Shiotani and Tsuge 1995), and *Ophiostoma novo-ulmi* (Bowden, Smalley *et al.* 1996) using a gene that confers resistance to the eukaryotic antibiotic hygromycin B1, the *E. coli* hygromycin B phosphotransferase gene (*hph*) under the control of different promoters (*hph* cassette). The replacement is made possible by flanking regions around the *hph* cassette that are homologous to regions of the gene being targeted. The resistance gene is integrated into the genome by homologous recombination into the targeted gene. This stops the wild type gene from functioning.

Targeted gene disruption has proven to be more difficult in filamentous fungi compared to yeast. Shiotani (1995) investigated systematically homologous recombination and gene targeting in the filamentous fungus *A. alternata* and found that both linear and circular molecules are successfully integrated into the genome. When using circular molecules the frequency of gene targeting was proportional to the length of the homologous sequence ((Shiotani and Tsuge 1995); (Bird and Bradshaw 1997)).

Many fungal species produce a toxin thought to have a pathogenic effect on the host plants. To study the pathogenicity of the toxin, toxin deficient mutants have been generated with both UV mutagenesis and targeted gene disruption. For example, some species of *Fusarium* infect a wide range of plants and in some cases pathogenicity is thought to be caused by the production of the phytotoxin enniatin. To test the hypothesis that enniatin contributes to *Fusarium* 's pathogenicity, an enniatin biosynthetic gene in a virulent *Fusarium avenaceum* strain was disrupted. It was found that the non-producing enniatin synthetase strains had a

decreased virulence showing that enniatin production does contribute to *F. avenaceum*'s pathogenicity (Herrmann, Zocher *et al.* 1996).

Cercospora kikuchii is a pathogenic fungus that infects soybeans causing the disease purple seed stain. Evidence suggests that the toxin produced by *C. kikuchii*, cercosporin, is responsible for the pathogenicity of the fungus. Cercosporin is light activated and light has been shown to be important in the disease development. Direct treatment of soybean tissue with pure cercosporin resulted in disease symptoms and cercosporin has been isolated from infected plant lesions. Mutants in the cercosporin biosynthetic pathway failed to produce cercosporin. These mutants were shown to be non-pathogenic to soybean and show that the toxin appears to play a vital role in the pathogenicity of the fungus (Upchurch, Walker *et al.* 1991).

Examples also exist of toxin deficient mutants that retain wild type pathogenicity. *Ophiostoma novo-ulmi* is a fungus that infects the xylem and is pathogenic to elm trees. It has been proposed that the protein cerato-ulmin (CU) produced by *O. novo-ulmi* is a factor in dutch elm disease. CU^- mutants of *O. novo-ulmi* were made by targeting a gene involved in CU production (Bowden, Smalley *et al.* 1996). The CU^- mutants, although not producing CU, still had wild type pathogenicity to elm trees suggesting the CU didn't have a direct role in pathogenic effects of the fungus.

Fusarium solani and its teleomorph *Nectria haematococca* is a phytopathogenic fungus that invades pea plants causing foot rot of pea. A longstanding hypothesis is that the cutinases produced by such fungi play an integral part in infection of the plant by breaking down the plant cuticle so the fungus can invade the host. Two experiments, both using targeted gene replacement, generated cutinase deficient (*cuta*$^-$) mutants ((Crowhurst, Binnie *et al.* 1997), (Stahl 1992)). In both cases, the *cuta*$^-$ mutants had wild type virulence indicating that neither the *cuta* gene or cutinase are not required for lesion development and pathogenicity of the fungus.

Disrupting known genes by gene targeting can also give conclusive evidence that the putative biosynthetic gene is on the toxin pathway. An example of gene disruption being used for confirmation of biosynthetic action is the *stcU* gene in *A. nidulans*. The gene was

thought to be involved in the ST biosynthetic pathway due to its sequence similarities with the *A. parasiticus ver-1* gene. The *stcU* gene was disrupted by gene targeting and the successful *stcU*$^-$ mutants were found to produce versicolorin A but not ST. The results confirmed the hypothesis that the *stcU* gene encoded an enzyme necessary for converting versicolorin A to ST (Keller, Kantz *et al.* 1994).

To confirm that the *dotA* gene is on the *Dothistromin* biosynthetic pathway, *dotA*$^-$ mutants need to be made and dothistromin production examined. *phn1* is homologous to *dotA* and in a *dotA*$^-$ mutant, could compensate for the loss of functional DOTA. If *dotA*$^-$ mutants continue to produce dothistromin, then targeted disruption of *phn1* should take place in *dotA*$^-$ mutants and in wild type to determine the role of *phn1* on the dothistromin biosynthetic pathway.

If *dotA*$^-$ mutants are also dothistromin$^-$ mutants, the dothistromin biosynthetic pathway intermediates present need to be examined. These intermediates will give us an indication at which step on the dothistromin pathway DOTA functions.

1.6 AIMS AND OBJECTIVES

The main aims of this research are to investigate the function of putative dothistromin genes:

The putative dothistromin biosynthetic gene *dotA* will be disrupted. *dotA*$^-$ mutants will allow evaluation of the proposed dothistromin biosynthetic pathway and further investigation of the shared characteristics of the AF/ST and dothistromin biosynthetic pathway. Mutants will be assessed to see if they are dothistromin deficient and whether they accumulate versicolorin B as expected.

The clone λBMKSA will be sequenced further to characterise *pks*^{dot} in preparation for disruption of this gene. The clone will also be investigated for the presence of other putative dothistromin biosynthetic genes.

2.0 METHODS AND MATERIALS

2.1 FUNGAL AND BACTERIAL STRAINS, LAMBDA CLONES AND PLASMIDS.

Fungal and bacterial strains, Lambda (λ) clones and plasmids used in this study are listed in Table 2.1.

2.2 MEDIA

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

2.2.1 Luria Broth (LB).

(g/L): tryptone, 10; NaCl, 5; yeast extract, 5.

2.2.2 LB solid media.

(g/L): tryptone, 10; NaCl, 5; yeast extract, 5; agar,15.

2.2.3 Selective LB media.

Supplements added at the following concentrations.
(μg/mL): ampicillin, 100; isopropylthio-β-D-galactoside (IPTG), 30; 5-bromo 4-chloro 3-indolyl-β-D-galactoside (X-gal), 60.

2.2.4 *D. pini* media (DM).

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

2.2.5 *D. pini* Broth (DB).

(g/L) : nutrient broth (Oxoid), 23; and Malt extract (Oxoid), 50.

2.2.6 *D. pini* Sporulation Media (DSM).

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

Table 2.1 Lambda (λ) clones, plasmids, Fungal and Bacterial Strains.

Strain, Plasmid, λ Clone.	Relevant Characteristics	Source or Reference.
Fungal Strain		
Dothistroma pini		
Dp002 aka NZE1	Single spore isolate, laboratory strain.	Long mile Road, NZFRI, Rotorua, New Zealand.
3-3-1-1A aka NZE 5	Single spore isolate, laboratory strain.	Kinleith, New Zealand (Hirst 1997).
Bacterial strains.		
Escherichia coli.		
XL1-Blue	SupE44 hsdR17 recA1 endA1 gyrA46 *thi rel* A1 lac$^-$ F' [proAB$^+$ lacI^{q}Δ (lacZ) M15 Tn10 (tet^r)]	(Bullock 1987)
λ clones		
λCGVI	LambdaGEM-12 (Promega) containing genomic DNA (including *dotA*) from DP002.	(Gillman 1996)
λCGV2	LambdaGEM-12 (Promega) containing genomic DNA (including *phn1*) from DP002.	(Gillman 1996)
λBMKSA	LambdaGEM-12 (Promega) containing genomic DNA (including *pks1*) from DP002.	(Morgan 1997)
Plasmids		
pAN7-1	6.5 kb HmB^R Amp^R	(Punt and van den Hondel 1992)
pCWhyg	5 kb HmB^R Amp^R	C. Wasmann, University of Arizona.
pGEM-T	Amp^r, lacZ' (3.0 kb)	Promega.
pR150	PUC18 containing a 0.9 kb EcoRI-BamHI fragment from λCGV1.	(Monahan 1998)
pR153	pUC based plasmid containing 1.1 kb subclone from λCGV1.	(Monahan 1998)
pR156	pUC based plasmid containing 2.4 kb subclone from λBMKSA.	(Morgan, 1997)
pR163	pUC based plasmid containing 3.5 kb subclone from λBMKSA.	(Laarakkers, 1999)
pR181	pUC based plasmid containing 0.9 kb subclone from λBMKSA.	(Laarakkers, 1999)
pR204	pGEM-T based plasmid containing 1.3 kb insert of *dotA* flanks.	This study
pR208	*dotA* disruption vector.	This study
pR209	pGEM-T based plasmid containing 0.24 kb subclone from λBMKSA.	This study
pR210	pGEM-T based plasmid containing 0.3 kb subclone from λBMKSA.	This study

2.2.7 Selective DSM.

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

2.2.8 *D. pini* Top media (DM Top).

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

2.2.9 Osmotically Stabilised DM (DM Suc).

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

2.2.10 Aspergillus Minimal Media + 2% glucose (AMM).

(g/L): $NaNO_3$, 6.0; $MgSO_4.7H_2O$, 0.52; KCL, 0.52; KH_2PO_4, 1.52; $FeSO_4.7H_2O$, trace; $ZnSO_4.7H_2O$, trace; 2% glucose.

2.3 GROWTH AND MAINTENANCE OF CULTURES.

2.3.1 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 (section 2.2.3) Plates were sealed with parafilm and stored at 4°C. All cultures were regularly subcultured by streaking onto fresh LB plates. All transformed *E. coli* were also mixed with sterile glycerol at a *E. coli* (overnight inoculated LB broth) to glycerol ratio of 17:3 and stored at –80°C.

2.3.2 Growth and Maintenance of *D. pini* cultures.

Subcultured *D. pini* cultures were grown on DM or DSM plates at 22°C in ambient light for 14-28 days.

2.3.3 Single Spore Purification.

In order to get a genetically pure colony of *D. pini*, single spores were isolated and grown as described below.

A sterile loop of milli-Q water was wiped over the colony (growing on DSM) to be purified. This loop was then streaked across a new DSM plate (selective in the case of transformants), the plate was turned 30° and streaking 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.

2.3.4 Growth of *D. pini* from transformed protoplasts.

D. pini protoplasts were embedded in 5 mL molten DM top agar and poured onto 20 mL plates of DMSuc. Putative transformants and negative control plates had 70 μg/mL hygromycin in 5 mL DM top agar added 24 hrs after embedding of protoplasts. Protoplasts were grown for 14 days at 22°C. Colonies growing on hygromycin were subcultured and grown on DSM for 14 days before finally subculturing onto DSM/70 μg/mL hygromycin.

2.4 COMMON BUFFERS AND SOLUTIONS.

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

2.4.1 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.

2.4.2 1 x TAE Buffer.

40 mM Tris-HCl, 2 mM Na_2EDTA and 20 mM acetic.

2.4.3 1 x TBE Buffer.

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

2.4.4 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.

2.4.5 RNAse A (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-Cl (pH 7.4) and store at -20°C.

2.4.6 Gel Loading Buffer (10x).

2 M Urea, 50% (v/v) glycerol;, 50 mM Tris acetate, o.4% (w/v) bromophenol Blue and 0.4% (w/v) Xylene cyanol.

2.4.7 OM buffer.

1.6M $MgSO_4.7H_20$, with 10mM Na_2HPO_4/100mM $Na_2H_2PO_4$ buffer (pH 5.8).

2.4.8 ST buffer.

1.0M sorbitol, 100mM Tris-HCl (pH 8.0).

2.4.9 STC Buffer.

1.2M sorbitol, 50mM Tris-HCl (pH 8.0), 50mM $CaCl_2$.

2.4.10 Chemiluminescent Detection Reagents:

Buffer 1

100 mM Maleic Acid, 150 mM NaCl. pH 7.5

Washing Buffer

0.3 % Tween-20 in buffer 1 (section 2.4.10.1).

Buffer 2

1% blocking reagent (Roche), in buffer 1.

Buffer 3

100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$. pH 9.5.

Substrate Solution

CSPD Lumigen stock solution (Roche) diluted 1:100 in Buffer 3 (section 2.4.10.4)

2.4.11 Reagents for Southern Blotting.

Denaturing Solution.

500 mM NaOH, 500 mM NaCl.

Neutralising Solution.

500 mM Tris (pH 7.4), 2 M NaCl.

2x SSC.

0.5 M NaCl, 0.03 M sodium citrate.

Wash Solution One.

2x SSC, 0.1% SDS.

Wash Solution Two (1x SSC).

0.25 M NaCl, 0.015 M sodium citrate.

2.5 GENOMIC DNA ISOLATION FROM *D. PINI* CULTURES.

(Al-Samarrai and Schmid 2000)

30 mg of freeze dried mycelia was ground to a fine powder with liquid nitrogen and sterile mortar and pestle. The powder was resuspended in 500µl of freshly prepared lysis buffer (Tris-acetate (pH 7.8), 40mM; Na_2EDTA, 20 mM; SDS (w/v) 1%) and mixed with VIGOROUS pipetting. 2 µl of 10mg/ml RNAse A was added and mixture was incubated for 5 min at 37°C. 165 µl of 5M NaCl was added, to precipitate cellular debris and mixed by inversion. Mixture was then centrifuged at 4 °C (13, 000 rpm in a microcentrifuge) for 20 minutes. The supernatant was transferred immediately into a fresh tube. 400µl chloroform and 400 µl of phenol was added and the tube inverted until solution became milky (usually >50 times) This was then centrifuged as before for 20 minutes. The chloroform/phenol step was repeated to the aqueous phase each time until the interface became almost indistinguishable. The aqueous (top) phase was removed had one volume of chloroform added and centrifuged for 10 minutes. The top layer containing the DNA was then precipitated in 2 volumes of ice cold 95% ethanol and centrifuged at 13, 000 g for 5 minutes. The pellet was washed three times with 500 µl of 70% ethanol, dried and resuspended in 50 µl of TE.

2.6 PURIFICATION OF DNA.

2.6.1 Agarose Gel Purification of DNA fragments.

DNA containing the fragment to be purified was run on a 1.5% SeaPlaque (FMC) low melting point agarose gel in 1x TAE buffer (Section 2.4.2). After staining in ethidium bromide (Section 2.4.4), the DNA was visualised under long wave UV light, 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 manufacturers instructions.

2.6.2 Purification of PCR Products from a PCR Reaction.

DNA was purified from a PCR reaction either by; gel purification (Section 2.6.1), or the QIAquick PCR purification Kit (Qiagen) according to manufacturers instructions.

2.6.3 Purification of Plasmid DNA from *E. coli.*

Plasmid DNA was isolated from *E. coli* using a QIAGEN plasmid minikit ($\leq$20μg plasmid DNA) or QIAGEN plasmid midikit ($\leq$ 100μg plasmid DNA) according to manufacturers instructions. Freshly grown colonies of transformed *E. coli* were used to inoculate LB broth containing the appropriate selective antibiotic. The inoculated broth was then incubated at 37°C overnight or according to instructions accompanying the kit before plasmid isolation was able to proceed.

2.7 DETERMINATION OF DNA CONCENTRATION

DNA was quantified by one of the following methods.

2.7.1 Determination of DNA concentration by fluorometric assay.

Fluorometric quantification was used for pure or impure DNA samples of concentrations thought to be higher than 100 ng/μL. DNA was quantified on a Hoefer Scientific TKO 100 Flurometer according to the manufacturers protocol. The scale of the Fluorometer was set to 100 using 2 μl of 100 μg/mL calf thymus DNA added to 2 mL of a dye solution containing 1xTNE buffer (10mM Tris-HCl, 1mM Na_2EDTA and 100mM 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 (if there was enough sample DNA, the assay was performed in triplicate).

2.7.2 Determination of DNA concentration by Gel Electrophoresis.

Determination of DNA concentration by gel electrophoresis was used for DNA samples thought to be lower than 100 ng/μL. A series of Lambda DNA or pUC118 DNA concentration standards were run 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. This method was performed for most samples as it confirmed the presence of DNA and determined if more accurate quantification was required.

2.8 RESTRICTION ENDONUCLEASE DIGESTION OF DNA.

Restriction digests were performed at 37°C, in the buffer specified by the manufacturer, for 2-3 hours. Digestion was performed with 1-10 units of restriction enzyme per μg DNA. RNA was removed by the addition of RNAseA (section 2.1.5.) to a final concentration of 1.0 μg/mL prior to incubation. Digests were checked by running an aliquot on an agarose gel (section 2.9) after heating at 65°C for 5 minutes. If digestion was not complete, further restriction enzyme was added to the digestion mixture and incubation extended for 1 hour when the samples were checked for completion of digestion.

2.9 AGAROSE GEL ELECTROPHORESIS OF DNA.

DNA fragments were size fractionated by electrophoresis through 1% -1.5% agarose dissolved in 1 x TAE (section 2.1.2) or TBE (section 2.1.3) buffer at 60-100 volts. Dyes present in the gel loading buffer (section 2.1.6), which was added to DNA samples before loading, allowed estimation of DNA migration. After electrophoresis, agarose gels were stained in ethidium bromide for 10-30 minutes, washed in water, observed under short wave UV light and photographed.

2.10 DETERMINATION OF DNA MOLECULAR WEIGHTS.

DNA fragments were sized by running the DNA sample on an agarose gel alongside known size ladders such as; *Hind*III/*Eco*RI double digest of lambda DNA, 1kb ladder or 1 Kb+ ladder (Life Technologies). 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.

2.11 DNA SUB-CLONING TECHNIQUES.

Fragments to be sub-cloned were purified (section 2.6.1) usually by gel purification, then ligated into the appropriate vector according to Section 2.11.1 or manufacturers instructions, and transformed into a suitable *E. coli* host strain (section 2.11.2). Blue/white colour selection was used for initial screening of transformants. When numbers of suspected transformants were large, PCR (section 2.13.3) using appropriate primers or the digestion using clonechecker kit (Life Technologies) was performed to further screen transformants. The presence of insert was always confirmed by restriction enzyme digestion (section 2.8) or PCR (section 2.13) with isolated plasmid DNA (Section 2.5.1).

2.11.1 Ligation Reactions.

Ligation reactions were set up in 20 µl with the required vector : insert ratio and final concentration of 1x ligation buffer and 40u/µl of T4 ligase. In the case of insertion into a single restriction site, the vector was SAP treated (Section 2.11.2) prior to ligation reaction. Background (with SAP treated vector only) and ligation (with non-SAP treated digested vector) controls were also ligated. All mixtures were mixed well and pulse spun. 5 µl of the ligation mixture was removed and had 5 µl of gel loading buffer added prior to addition of T4 ligase as a before ligation sample. All mixtures were incubated at 4°C overnight after thorough mixing. Ligations were run on a gel to check completion. Ligation mixtures and controls were used to transform *E. coli* (method 2.12.1.2) and left to incubate at 37°C overnight. These *E. coli* were plated on LB/Amp/Xgal/IPTG media and transformants were identified. Selective transformant plates were stored at 4°C until needed.

2.11.2 Shrimp Alkaline Phosphatase (SAP) treatment of digested DNA

1 - 2 µl (1-2 units) of SAP (Roche) was added to 34 µl of clean digest and 4 µl of 10x SAP buffer. This mixture was incubated at 37°C for 30 - 50 minutes. The enzyme was inactivated at 65°C for 15 minutes.

2.12 TRANSFORMATION PROTOCOLS

2.12.1 Transformation of *E. coli* by electroporation.

2.12.1.1 Preparation of Electroporation Competent *E. coli* Cells.

One litre of LB broth (Section 2.2.1) was inoculated with 10ml of an overnight culture of the desired *E. coli* strain XL-1), and grown at 37°C, with vigorous shaking to mid-log phase (OD_{600} 0.5-1.0). The cells were chilled on ice for 20min then harvested by centrifugation for 10 min at 4000 g (all centrifugation steps performed at 4°C). The cells were washed (by resuspension, centrifugation at 4000 g to pellet the cells and removal of the supernatant) in 1.0 litre and 0.5 litre of ice cold sterile water, 20ml of ice cold 10% (v/v) glycerol, than finally resuspended in 4 mL ice cold 10% (v/v) glycerol. Cells were stored at –80°C in 40µl aliquot's.

2.12.1.2 Electroporation of Competent *E. coli* Cells.

2 µl of DNA (ligation mixture, controls or plasmid) was added to 40 µl of electroporation competent *E. coli* cells (section 2.12.1.1) in an 0.2 cm ice cold electroporation cuvette, gently mixed and tapped to the bottom. The cells were electroporated in a Biorad gene pulser set at 25µF, 2.5 kV and 200Ω. 200 µl of LB broth was immediately added to the cells which were transferred into an eppendorf and incubated with shaking at 37°C for 10 – 30 min. Positive (circular plasmid DNA) and negative (water only) controls were always included. Cells were spread onto LB agar containing appropriate supplements (Section 2.2.3) and incubated overnight at 37°C.

2.12.2 Transformation of *D. pini* protoplasts.

2.12.2.1 Preparation of competent *D. pini* protoplasts.

100µl of freshly grown *D. pini* mycelium ground in sterile milli Q water, was spread onto cellophane covered DM media The cellophane plate was incubated in the dark for 6 days at 22°C. After 6 days, a light covering of mycelium had grown on the cellophane. The cellophane was stripped of the media placed mycelia down in a sterile petri dish containing 10ml of sterile 15mg/ml Glucanex (beta-glucanase, Chemcolour Industry, NZ) dissolved in OM buffer (Section 2.4.7). The petri dish was sealed with parafilm and left shaking in 37°C for 3-4 hours. Protoplast formation was confirmed by placing 20 µl of the incubated solution on a microscope slide and examining at 400x. 5ml of the incubated protoplast solution was put in a 15ml corex tube, overlaid with 1ml ST buffer (Section 2.4.8) and centrifuged at 4°Cfor 5 minutes at 1085g. The protoplasts forming a white band at the interface of the two solutions, were removed and washed twice in 5ml STC buffer (Section 2.4.9) being pelleted in between washes by centrifuging as above. The pellet was resuspended in 200µl STC buffer and stored at 4°C for no longer than 4 hours before use. The concentration of the protoplasts was determined by counting with a haemocytometer.

2.12.2.2 Transformation of *D. pini* protoplasts.

For each transformation, between $5x10^6$ and $1x10^7$ protoplasts in 150µl of STC buffer were mixed with 5µg of DNA and incubated at 22°C for 20 minutes. In three steps 250, 250 and 850 µl of 40 % Polyethyleneglycol (PEG) 6000 solution in STC buffer (Section 2.4.9) were carefully mixed with DNA/protoplast mixture and the final suspension was incubated for a further 20 minutes at 22°C. The suspension was then diluted in 5ml of STC buffer and the protoplasts were collected by centrifugation (4 °C, 10 min, 3000rpm (1085 g) SS34) and resuspended in 500µl of STC.

100µl of this suspension was put into 5ml DM top agar (section 2.2.8) and overlayed onto plate containing 20ml DMSuc (Section 2.2.9). Selective antibiotics (in most cases 70µg/mL hygromycin) were added 24hrs later by overlaying the plates with 5ml DM top agar to which the appropriate concentration of antibiotic had been added.

2.13 AMPLIFICATION OF DNA BY THE POLYMERASE CHAIN REACTION (PCR).

Due to the nature of the research, a number of different PCR reactions were used.

PCR reactions were set up on ice using a master mix which contained all common reagents for the PCR reactions. Uncommon reagents were pipetted separately. PCR reactions were in a total volume of 25 µl in 0.2 mL PCR tubes. Negative controls of no DNA, only one primer and a positive control were included in each experiment. The reactions were stored at -20 °C. All reactions used *taq* polymerase and *taq* buffer from Life Technologies.

2.13.1 Reagents and Cycling Conditions for Basic PCR.

The final concentrations of each component in 1 reaction were, 1 x *taq* buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit *taq* DNA polymerase, 0.2µM of each primer and 1-3 ng of plasmid template or 20 ng of genomic template.

After mixing, the PCR reaction tubes were placed in a thermal cycler (Corbett FTS-960) preheated to 94°C. The samples were then subjected to an initial denaturation step of 1 min at 94 °C, then 30 cycles of denaturation at 94 °C for 15 sec, annealing at 55 °C for 30 sec (adjusted depending on primers used and specificity wanted), extension at 72 °C for 90 sec (depending on expected product size).

2.13.2 Reagents and Cycling Conditions for amplification of combined *dotA* flanks.

The final concentrations of each component in 1 reaction were, 1 x *taq* buffer, 3.0 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit *taq* DNA polymerase, 4µM primer dkr1B, 4µM primer dkr1C, 5 ng of 5' flank and 5 ng 3' flank.

After mixing, the PCR reaction tubes were placed in a thermal cycler (Corbett FTS-960) preheated to 94°C. The samples were then subjected to an initial denaturation step of 1 min at 94 °C, then 30 cycles of denaturation at 94 °C for 15 sec, annealing at 50 °C for 30 sec extension at 72 °C for 90 sec.

2.13.3 Reagents and Cycling Conditions for amplification from *E. coli* colonies.

The final concentrations of each component in 1 reaction were, 1 x *taq* buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit *taq* DNA polymerase, 0.2μM of each primer and 1-3 ng of plasmid template or 20 ng of genomic template.

After mixing, the PCR reaction tubes were placed in a thermal cycler (Corbett FTS-960) preheated to 94°C. The samples were then subjected to an initial lysis/denaturation step of 2 min at 94 °C, then 30 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec extension at 72 °C for 60 sec and a final extension step at 72 °C for 4 min.

2.13.4 PCR of DNA from Fungal Mycelium.

Based on a protocol from the fungal genetics stock centre web site.
Ferreira, Adlane V. B. and Glass, N. Louise- Biotechnology Laboratory and Department of Botany, University of British Columbia, Vancover, Canada, V6T1Z4.

A small mycelial chunk (2mm by 2mm) of *D. pini* was placed into a 500μl Eppendorf tube
Microwave on high power for 5 minutes (with beaker of water in microwave)
Add 30μl sterile H_2O and vortex. Spin @ 13000 rpm for 5 minutes in microcentrifuge.
Collect supernatant and put into new tube. Use 5 μl of this in 25μl PCR reaction with the following reagents: 10mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 % Triton X-100, 5% glycerol, 0.2 mM dNTPs, 0.2μM primer, 2 units of taq polymerase.

After mixing, the PCR reaction tubes were placed in a thermal cycler (Corbett FTS-960) preheated to 94°C. The samples were then subjected to an initial lysis/denaturation step of 1 min at 94 °C, then 30 cycles of denaturation at 94 °C for 15 sec, annealing at 55 °C for 30 sec, extension at 72 °C for 90 sec and a final extension step at 72 °C for 90 sec.

2.13.5 PCR amplification of ITS1 region.

This protocol is based on one described in Bradshaw *et al* 2000. Each PCR reaction was made up in a total volume of 50μl.

1x PCR buffer, 1.5 mM $MgCl_2$, , 0.2 mM dNTPs, 0.2μM primer, 0.3 units of *taq* polymerase and 20 – 50 ng of template DNA.

After mixing, the PCR reaction tubes were placed in a thermal cycler (Corbett FTS-960) preheated to 95°C. The samples were then subjected to an initial denaturation step of 4 min at 95 °C, then 35 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 1 min and a final extension step at 72 °C for 4 minutes.

2.14 DNA SEQUENCING

Automatic sequencing reactions were performed using the ABI PRISM ™ BigDye ™ Terminator Cycle Sequencing Ready Reaction Kit version 1 (Perkin Elmer) with 200 ng plasmid DNA or 2ng/100bp of PCR product, 3.2 pmol primer, and 8 µl of terminator ready reaction mix in a total volume of 20 µl Samples were run on an ABI 373 and 377 automatic sequencer.

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* (1993).

Microtitre wells were coated (100 µl/well) with Doth-MSA conjugate diluted in phosphate-buffered saline (PBS). 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% Ovalbumin (OVA) 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 contained equal volumes of 2x PBST (+2% OVA) and AMM + 2% medium.

Macerated mycelium was used to inoculate 25ml of AMM + 2% glucose and incubated at 24°C while shaking for 7 and 9 days. 200 µl samples were removed form the flasks and centrifuged at 13000 rpm 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 (2x PBST, 2% OVA, 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 AMM + 2% media.

Standards and samples, 100 µl of each, were pre-incubated at 37°C for 1 hour with 100 µl of labelled peroxidase, per 10C12 (1:30000 in dilution buffer). Aliquot's were then transferred to the washed Doth-MSA microtitre plates and incubated at 37°C for 3 hours. After the incubated, the plates were washed six times in PBST to remove any free labelled peroxidase. 200µl of freshly prepared substrate mixture (40 mg *o*-phenylene diamine, 0.51 g citric acid, 40µl of 30% H_2O_2, 27.7 mL 0.2 M Na_2HPO_4, made 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 min. The peroxidase reaction was stopped by the addition of 50 µl of 4M sulphuric acid to each well. Absorbance was measured at 492 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 SOUTHERN BLOTTING AND HYBRIDISATION

2.16.1 Southern Blotting (Capillary).

The DNA blotting method used was based on that of Southern (1975). DNA to be transferred onto a nylon membrane was separated by gel electrophoresis, through a 1-1.5% agarose gel, stained in ethidium bromide (Section 2.4.4) and photographed with a ruler alongside the gel. The gel was gently agitated in 250mM HCL for 15 minutes to de purinate the DNA so that high molecular weight regions would transfer efficiently. The DNA in the gel was then denatured by gentle agitation for 30 minutes denaturing solution (section 2.4.11.1) for 30 minutes. The gel was neutralised by washing twice in neutralising solution

(section 2.4.11.2) for 20 minutes per wash. Before placing on the blotting apparatus, the gel was washed in 2 x SSC (section 2.4.11.3) for 5 minutes. The blotting apparatus was constructed as described by Ausubel *et al* (1994). 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. The membrane was stored between two sheets of acetate at 4°C.

2.16.2 DIG labelling of DNA probe

DNA needed for probing the membrane was labelled with Digoxygenin (DIG)-11-dUTP with a DIG-random labelling kit (Roche) according to manufacturers instructions.

2.16.3 Southern Blot Hybridisation of DIG Labelled Probe.

The membrane to probe was placed in hybridisation tube and washed with 50ml of 2x SSC for a few minutes. The 2 x SSC was gently poured out and 20 mL of DIG Easy Hyb solution (Roche) (20ml/100 cm^2 of membrane) was added to the tube. The membrane was placed in a rotary oven at 40°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 35 mL of fresh DIG Easy Hyb solution that contained 2.5 ng of the denatured probe per mL of DIG Easy Hyb. The contents of the hybridisation tube were mixed briefly and incubated over night at the hybridisation temperature ($T_{opt} = T_m - 20$ to 25°C, where $T_m = 49.82 + 0.41(\%G + C) - (600/l)$ and l = length of hybrid in base pairs).

After overnight incubation, excess probe was washed off the blot as follows.
The membrane was removed from the hybridisation tube with forceps and placed in 200 mL of wash solution one (section 2.4.11.4) (prewarmed to 68°C) and incubated on a shaking platform at 68°C for 5 minutes. Wash solution one was replaced with 200 mL of fresh warmed wash solution one and incubated on a shaking platform at 68°C for 5 minutes.

Wash solution one was replaced with 200 mL of warmed wash solution two (section 2.4.11.5) and incubated on a shaking platform at 68°C for 15 minutes. This step was

repeated with 200 mL of wash solution two. The membrane was then wrapped in gladwrap stored at 4° C until needed for chemiluminescent detection.

2.16.4 Chemiluminescent Detection.

The membrane was washed for 1-5 minutes in washing buffer (section 2.4.10.2). Membrane was then incubated in buffer 2 (section 2.4.10.3) for 30 minutes and then incubated in Buffer 2 with dilute anti-digoxigenin-AP Fab 1:10,000 (Roche) for 30 minutes. Membrane was washed twice for 15 minutes in washing buffer and then equilibrated for 1 – 5 minutes in buffer 3 (section 2.4.10.4).

The wet membrane was placed onto acetate paper with the DNA side facing up. 1 mL substrate solution lumigen (section 2.4.10.5) was dispensed over the surface of the membrane and a second acetate sheet was placed over top. Lumigen was spread evenly and gently over the surface and left for 1 minute. The top acetate sheet was then wiped firmly to remove the excess lumigen from membrane. The membrane was left between the two acetate sheets with the thin layer of lumigen for 5 minutes. The membrane was then removed form the acetate and blotted on clean 3MM filter paper and placed between two fresh sheets of acetate and incubated for 15 minutes at 37°C. The membrane was then exposed to Xray film (Kodak Scientific Imaging). for 3 hours.

2.17 ISOLATION OF SECONDARY METABOLITES FROM MYCELIUM OF *D. PINI*.

Macerated mycelia (10 x 20 mm /250ml) were incubated in AM media (2.2.10) for 9 days at 24°C.

The following protocol was repeated for each flask until the last step when the contents of all flasks were condensed into the same vial. One volume referred to below is equal to the original volume of media. Half a volume of acetone was added to mycelia and media and left for 30 minutes when half a volume of chloroform was added and contents of 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 a vacuum until all chloroform had boiled off.

The organic phase from all flasks of media were condensed into the same round flask until the last condensation when the last 5 mL of the chloroform/polyketide solution was filtered through filter paper into a smaller round flask and condensed. The small flask 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. 1 mL of chloroform was added to the small round flask and swirled until all of the polyketide was dissolved. This mixture was then glass vial and left in a fumehood (2-4 days) to evaporate the last of the chloroform.

2.18 DETECTION OF AFLATOXIN INTERMEDIATES.

Extracts prepared in 2.17 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 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.

Table 2.2 Sequencing Primers

Primer	Size (nt)	Tm[1] (°C)	Sequence (5' to 3')	Source/reference
pUC/M13 Foward	22	70	GCC AGG GTT TTC CCA GTC ACG A	Perkin Elmer
pUC/M13 Reverse	24	70	GAG CGG ATA ACA ATT TCA CAC AGG	Perkin Elmer
SP6	22	58	TTT AGG TGA CAC TAT AGA ATA C	Promega
T7	23	66	TAA TAC GAC TCA CTA TAG GGC GA	Promega
dmo1r	18	61.4	CGT CAG TGA TGA TGT CCG	This study
dmoR2	18	62.9	TGG CTG TGC TTC TTC TGG	This study
dmoR3	22	63.4	CGT TGA TAC TAC ACA AGC CTC G	This study
dmo1F	18	61.7	AGC AGG CAC TGA TTG AGG	This study
dmoF2	19	68.3	GTC TTC GAT GAC GCG GAGG	This study
dmoF3	22	61.1	CGA CAT AAT GAG ACT GTT CCA G	This study
dmoF4	20	63.6	CCT GAA GTC CGA AGT ATC GCA A	This study
R163ovlap	20	61.5	TTC CCA GAG GCA CAA AGC AC	This study
R163ovlpB	20	66.6	CGA TTG CGA TAC TGC TGT GG	This study
pksSP6C	17	60	GTT GTC GTT CCT GGT GG	This study
pksCA	20	63	CTT TCC TCG CTG TCT TCC TC	This study
pksCB2	18	59.9	TGA AGA AGT ATG TCG CCG	This study
PKSC3	19	50.4	GAA GAT GTC AAG TCT ATC C	This study
PKSC4	18	60.0	ACA GCA GTA TCG CAA TCG	This study
dmoCA	20	67.1	ATT ATG GAC GCC ACT GCT GG	This study
dmoCB	19	56.6	GCA GAA GAT GAA GGA AGA G	This study
dmoC3	18	61.4	CGG ACA TCA TCA CTG ACG	This study
JSfdkr	22	60.9	CAA TAC ATG GCA AAA GCT ACA C	This study
JSDkr1A	26	68	CGT TCT AGA GTC GCT CGC GAG GCG TA[2]	This study
JSDkr1B	27	65	GAA AAC GTT CCT CGC CGT CAT GGA GTA	This study
JSDkr1C	17	59	TCC TGC CCA TGG TGC GA	This study
JSDkr1D	26	60	GAC TCT AGA ACG AGT CTC AAT GTA TC	This study
pUChph9	18		CTC CAC GCG ACT ATA TAT	C. Young
pUChph7	18		ATT CCT GCA GAG ACC ATC	C. Young
JSphn1A	24	69.1	GGA GAT CTG AGG AAG TGT GCT TGG	This study
JSphn1B	21	65.7	CCA GAT CTT GGT CTC CAA TGC	This study
phn1EA	24	72.2	GGG AAT TCG AGG AAG TGT GCT TGG	This study
phn1EB	21	67.7	CCG AAT TCT GGT CTC CAA TGC	This study

[1] Melting temperature calculated as Tm(°C)=(A+T) +4(G+C)
From Itakura *et al.* (1984)

[2] Underlined bases indicate a restriction site.

pks K/O 1	24	69.9	GCA GAT CTG TGT TGG TCT CCA TCC	This study
pks K/O 2	24	69	ACA GAT CTG CTG GAG TGG ACC CTA	This study
JSphn1C	22	62.8	TGC GCT ATT ACA CTG GTC TAC C	This study
DPKS1	22	64	CTC GTT GAT TAT ACC CTT CTC C	Morgan (1997)
DPKS2	22	64	GGA GAA GGG TAT AAT CAA CGA G	Morgan (1997)
DPKS3	18	62	TAG GGT CCA CTC CAG CGG T	Morgan (1997)
DPKS4	18	62	ACC GCT GGA GTG GAC CCT A	Morgan (1997)
DPKS5	18	58	AAT AGC AAG CTG CAC GAC CAC TGG	Morgan (1997)
SLpks1	21	64	TGG AGG TGA TGC CAT CTT C	Larrakkers (1999)
SLpks2	21	64	GAC ATG TTG GCA AGT CTC TCA	Larrakkers (1999)
SLpks3	20	58	GCT TGC TAT TAC ATG CTT GC	Larrakkers (1999)
SLpks4	19	58	CTG CAT CAT GAG AAG GAC C	Larrakkers (1999)
R181fwdch	18	61.4	GCA CAA TGC GAC GGA ATG CT	This study
R181revch	18	66.5	CCA CTT CCA CTG CCA CTT	This study
Dst7ep7	21	64	ATT CGG CTA CAT GCC CTA CAC	Monahan (1998)
153Fep	23	70	GTC GAC GGA CAT TAT GGG AGA TG	Monahan (1998)

3.0 TARGETED REPLACEMENT AND CHARACTERISATION OF A PUTATIVE DOTHISTROMIN BIOSYNTHETIC GENE, *dotA*.

The *D. pini* gene *dotA* has been proposed as a dothistromin biosynthetic gene on the basis of amino acid sequence similarity (see section 1.4.3.; Monahan 1998). Targeted disruption of *dotA* will prevent assembly of a functional protein and allow investigation into the action of the gene product. The homologs of *dotA*, *ver-1* and *stcU* act in the conversion of versicolorin A in the AF and ST pathways respectively (Table 1.1 and figure 1.2) but versicolorin A has not previously been detected in *D. pini*. It is hypothesised that a non functional *dotA* gene will disrupt the dothistromin biosynthetic pathway at the conversion of versicolorin B to the next pathway product. Accumulation of versicolorin B in the *dotA*$^-$ mutants will confirm this hypothesis and absence of dothistromin will confirm the hypothesis that *dotA* is on the dothistromin biosynthetic pathway.

Targeted replacement of *dotA* requires optimisation of protoplast harvesting and transformation, construction of a *dotA* disruption vector and confirmation of targeted disruption.

3.1 OPTIMISATION OF *D. PINI* TRANSFORMATION SYSTEM.

A transformation system for *D. pini* has been developed (Bidlake 1996) but since the original development of the system, a number of changes have occurred which required the system to be optimised taking these differences into account.

Previous isolation of *D. pini* protoplasts used the cell wall digesting enzyme Novozyme. which has since gone out of production. A suitable alternative is another cell wall digesting enzyme Glucanex (beta-glucanase, Chemcolour Industry NZ).

The *D. pini* strain used for the original development of the transformation system was NZE1. NZE1 produces 0.1 ± 0.03 μg mg^{-1} mycelium of dothistromin after 14 days, compared with NZE5 which produces 0.7 ± 0.08 μg mg^{-1} (Ganley 2000). The strain NZE5 was selected to be transformed with the disruption vector due to its high level of dothistromin production.

The transformation system needed to be adjusted to accommodate these differences, also as regenerated protoplasts would be screened for a rare targeted integration event, the system needed optimising in order to yield as many protoplasts and transformants as possible.

3.1.1 Protoplast Formation and Regeneration.

Protoplast formation was optimised by altering different parameters including the concentration of the wall digesting enzyme, enzyme incubation periods and the growth of mycelia on solid or liquid media. The duration of the incubation and the concentration of the enzyme solution were both optimised using glucanex. An incubation time of 3-4 hours at 37°C in 15 mg/mL of glucanex (dissolved in OM buffer) was found to produce the highest yield of protoplasts.

Mycelium to be used for protoplast harvesting is commonly grown up in one of two ways. Mycelium can be grown in liquid culture (shake flasks) or spread onto a cellophane sheet on suitable solid media. A preliminary comparison of these methods suggested that the cellophane method yields a higher number of protoplasts (5.9×10^6/g wet weight mycelium) than the liquid grown mycelium (6.5×10^5/g wet weight mycelium). Moreover the liquid broth technique, although producing large amounts of mycelia, proved a difficult medium from which to separate the mycelial fragments from the protoplasts after the digestion with glucanex hence the cellophane method was used.

The regeneration frequency of wild type NZE5 protoplasts treated with PEG according to the transformation protocol (method 2.12.2.2) ranged from 1.2×10^{-3} to 8×10^{-3} (0.12 - 0.8 %, n=4).

3.1.2 Transformations.

The transformation system developed for *D. pini* uses positive selection of transformants based on hygromycin resistance. Previous studies have shown that 50μg/ml hygromycin B (HmB) is sufficient to inhibit the growth of wild type NZE1 (Bidlake 1996). 50μg/ml is also sufficient to inhibit wild type NZE5 growth (this study, results not shown). Two plasmids that confer hygromycin resistance are pAN7-1 and pCWhyg[3]. Both plasmids contain the *E. coli* hygromycin B phosphotransferase (*hph*) gene under the control of different promoters. pAN7-1 has the *hph* gene under the control of the *gpdA* promoter and the *trpC* terminator, both from *A. nidulans*. The hygromycin resistance cassette (*hph* cassette) in pCWhyg is constructed from an *A. niger glaA* promoter, *E. coli* hygromycin B phosphotransferase (*hph)* gene and the *trpC* terminator from *A. nidulans*. The *hph* gene, the promoter and the terminator from pCWhyg will be referred to from now on as the *hph* cassette.

pAN7-1 has been shown to confer hygromycin resistance to *D. pini* (Bidlake 1996) and was used as a positive transformation control. pCWhyg was required to construct the *dotA* targeted disruption vector (see figure 3.1) but had not previously been shown to confer hygromycin resistance on *D. pini*. Transformations to demonstrate the ability of the plasmid pCWhyg to confer hygromycin resistance were carried out using 5 μg of plasmid DNA (method 2.12.2.2). Data from three experiments show that the mean number of protoplasts transformed/μg DNA for pAN7-1 is 10.7 ± 1.5 and for pCWhyg is 9.0 ± 2.6.

In addition to the fast growing transformant colonies (4-9 mm after 14 days growth) other slow growing colonies (1-3 mm after 14 days growth) were also observed on HmB plates. The number of small colonies regenerating on selective plates is much smaller than the number of protoplasts regenerating on non-selective plates so the small colonies cannot be attributed to leaky growth (all over breakdown of HmB allowing non-resistant protoplasts to grow). The smaller colonies were spread evenly over the selective plates so did not appear to be satellite colonies (protoplasts regenerating in areas where the HmB has been

[3] Both pAN7-1 and pCWhyg plasmid maps are included in Appendix 1.

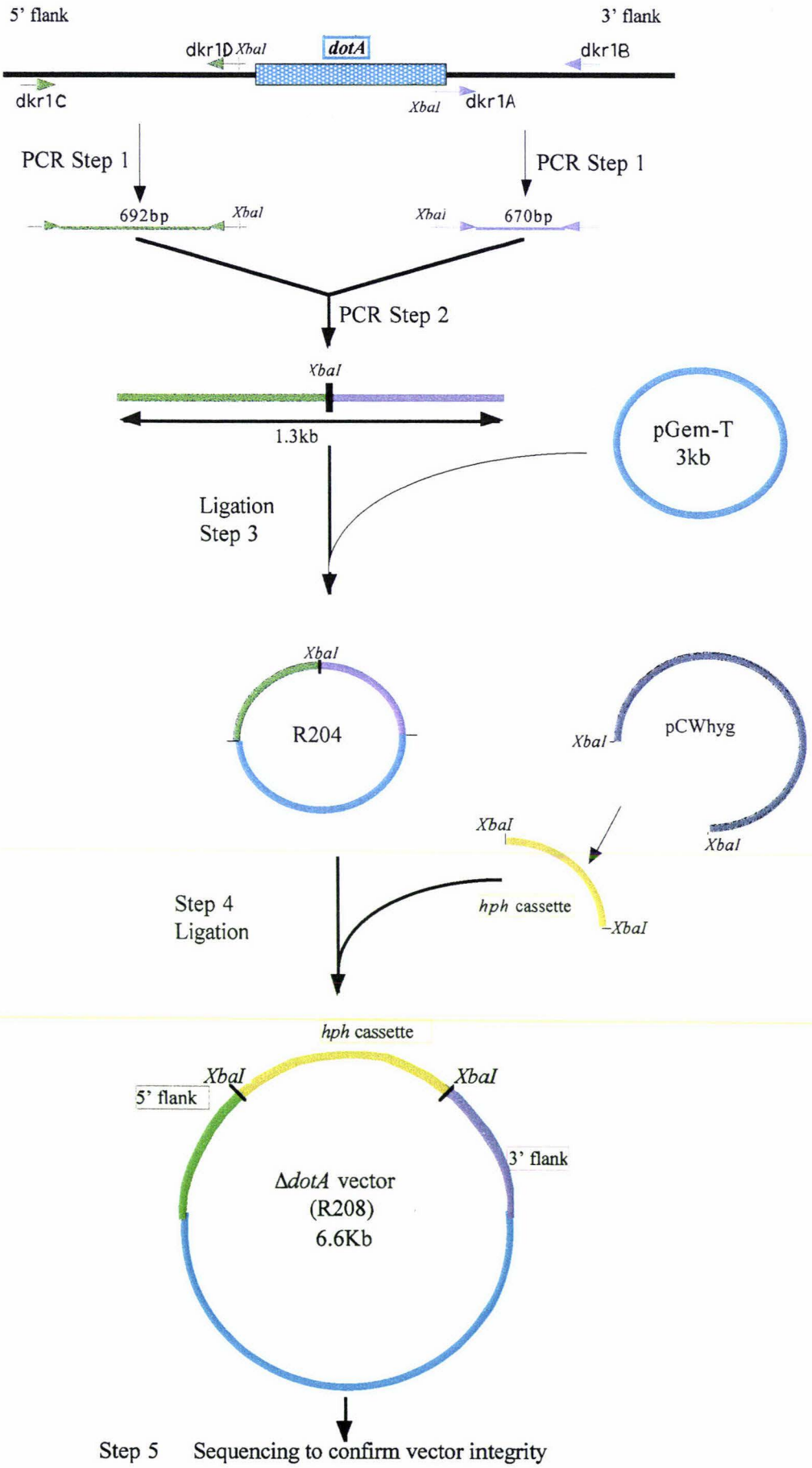
Figure 3.1 Schematic Diagram of *dotA* Disruption Vector Construction.

Steps 1-5 are described in the text. Positions of PCR primers dkr1A. dkr1B, dkr1C and dkr1D are indicated here and in Appendix 3.

 *dotA* coding region. genomic DNA flanking *dotA*.

Summary

Step 1: PCR amplification of 5' and 3' flanking regions.
Step 2: PCR amplification of 1.3kb flanking regions hybrid.
Step 3: Ligation of 1.3kb hybrid into pGEM-T plasmid.
Step 4: Ligation of *hph* cassette from pCWhyg into pR204 *Xba*I restriction site.
*Step 5:*Sequencing of disruption vector flanking regions.



Step 5 Sequencing to confirm vector integrity

broken down by the hygromycin phosphotransferase secreted by the transformants). The small colonies, when subcloned onto new DSM/70μg/ml HmB, did not continue to grow at all in comparison to the transformants that grew without impediment when subcloned onto HmB, hence appear to be abortive transformants (see discussion).

3.2 DISRUPTION OF *dotA* BY HOMOLOGOUS RECOMBINATION

The results described in section 3.1.3 show that pCWhyg is able to confer hygromycin resistance on *D. pini* and enables the *dotA* disruption scheme (Figure 3.1) using the *hph* cassette from the pCWhyg plasmid. The *dotA* gene was disrupted and the *dotA*$^-$ mutants examined. The technique widely used to disrupt selected genes in filamentous fungi is targeted disruption mediated by homologous recombination (section 1.5). In this project, gene disruptions were carried out by the transformation of a specific *dotA* disruption vector (pR208 for map see appendix 1) into wild type (strain NZE5) *D. pini* protoplasts.

3.2.1 Construction of *dotA* disruption vector pR208.

The disruption plasmid R208 was constructed as described below and in figure 3.1. All PCR conditions used are described in section 2.13.1. An alignment of the *dotA* coding region and the *dotA* disruption vector can be found in appendix 3, showing adjacent genes and primer positions.

Step 1 Amplification of 5' and 3' flanking regions.

The regions of homology for construction of the disruption vector needed to be chosen within the following parameters. Two sets of primers were designed to amplify 3' and 5' regions encompassing *dotA* and not including the conserved NADPH binding domain important to protein function. Primers were designed from these regions using Genejockey II primer design software which takes into account GC % and primer dimer formation. Both flanking regions were designed so the homologous recombination event would not interfere with other genes. The 3'flanking region was restricted in length due to the close proximity of the adjacent gene *ddh1* .

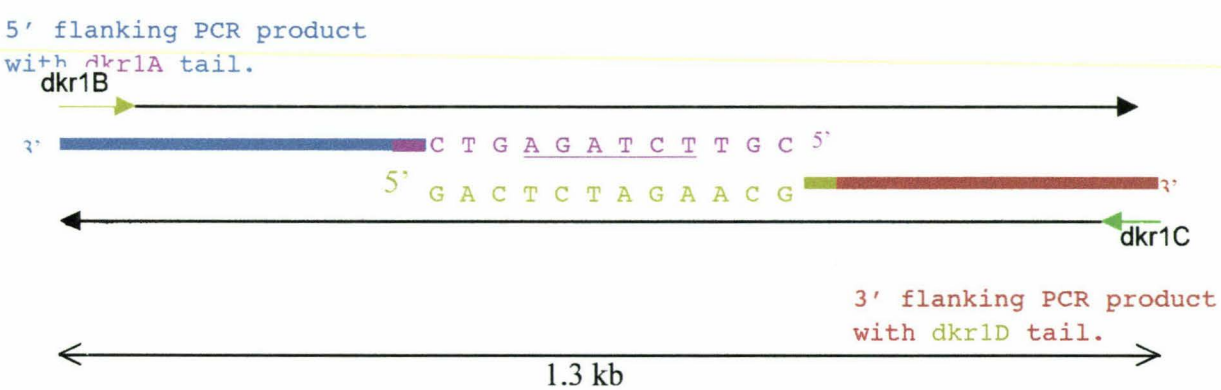
The 692 bp 5' flank of the *dotA* gene was amplified using primers dkr1C and dkr1D, from the template pR153 (a pUC based plasmid containing a subclone of λCGV1). The 670 bp 3' flank of *dotA* was amplified using primers dkr1A and dkr1B and *D. pini* genomic DNA (NZE1). PCR amplification of the 3' flank produced multiple amplicons. A 670 bp product was isolated by extraction from an agarose gel (section 2.6.1) and sequencing confirmed the identity of the product as the 3' flank.

The primers dkr1A (pink text) and dkr1D (yellow text) were designed to include an *Xba*I tail (underlined). The presence of a complementary tail on the end of the two original PCR products, facilitated hybridisation and amplification of both products together in step two (Figure 3.2).

Step 2 Combining the 5' and 3' flanking regions (Figure 3.2).

The two PCR products were purified (section 2.6.1) and these purified products were used in approximately equal amounts (5 ng of each flank per reaction) as the template for a PCR reaction using primers dkr1B (yellow arrow)and dkr1C (green arrow) to amplify a 1.3kb hybrid made possible by the complementary tails (*Xba*I site underlined) present on each of the amplified flanks. Black arrows show direction of PCR amplification from primers dkr1B and dkr1C in step two.

Figure 3.2 Hybrid Amplification of Two *dotA* flanking regions



It was necessary to optimise the [Mg^{2+}] in order to get a specific 1.3kb flanking region hybrid product. Large scale PCR amplification (section 2.13.2), followed by gel excision (section 2.6.1) produced a clean band of the desired 1.3kb product. Restriction digestions of the putative combined flanks PCR product were used to confirm it was the combined flanking region required for construction of the disruption vector.

Step 3 Ligation of 1.3kb hybrid into pGEM-T vector.

The 1.3kb flank PCR hybrid was ligated into a pGEM-T vector (Promega) by following the protocol recommended in the kit guidelines. *E. coli* colonies transformed with the ligation mixture were screened for recombinant plasmids with PCR (with the primers originally used to amplify the 1.3 kb hybrid and PCR conditions described in section 2.13.3) as well as with a screen using restriction digestion of plasmid DNA with a clonechecker kit (Life Technologies). Two colonies produced positive results for the 1.3 kb fragment.

The plasmid was extracted from one colony (section 2.6.3) and checked with a restriction digest using *Xba*I and *Sal*I. As *Sal*I cuts within the inserted fragment and the pGEM-T vector, it was possible to deduce the orientation of the insertion. The resulting plasmid was named pR204 (appendix 1).

Step 4 Ligation of hph cassette from pCWhyg into pR204 XbaI restriction site.

As pCWhyg has been shown to confer hygromycin resistance on *D. pini* (section 3.1.2) ligation of this *hph* cassette within flanking regions of the *D. pini dotA* gene produces a selectable disruption vector for the *dotA* gene.

pR204 was digested with *XbaI* and SAP treated (Section 2.1) to prevent religation of the plasmid. pCWhyg was digested with *XbaI* and the 2.3kb *hph* cassette was gel extracted (section 2.6.1). The *hph* cassette was ligated into the linear pR204 (section 2.2) and ligation products transformed into competent *E. coli* (section 2.12.1).

An initial PCR based screen was used identify the *E. coli* transformants containing the desired construct. Plasmids were isolated from some of the colonies (section 2.6.3) and digested with *Xba*I. Figure 3.3 shows that plasmid 6 contains the expected 4.3kb (vector) and 2.3kb (*hph* cassette) *Xba*I fragments and two other plasmids from colonies 1 and 7 did not produce bands expected for the correct disruption vector but produced one band of 2.6 kb. The plasmid from colony 6 was renamed pR208.

Step 5 Confirmation of pR208 integrity by sequencing.

Primers JS*dkr1*A, JS*dkr1*B, JS*dkr1*C and JS*dkr1*D, were used to sequence the *dotA* flanking regions and junctions in pR208 to ensure the sequence was correct. Sequencing also provided the orientation of the *hph* cassette within the flanks. A map of pR208 can be found in appendix 1.

Figure 3.3 Screen of ligation products for *dotA* disruption vector.

Agarose gel electrophoresis (section 2.9) was used to examine three plasmids for the desired *dotA* disruption vector construct. Plasmid 6 (lanes 4 and 5) was shown to contain the *Xba*I fragments (2.3kb and 4.3 kb) expected from the *dotA* disruption vector.

Lane	Enzyme	DNA	DNA fragment from *Xba*I digestion (kb)
2	*Xba*I	Plasmid 1	2.6
3	No Enzyme	Plasmid 1	
4	*Xba*I	Plasmid 6	2.3 and 4.3
5	No Enzyme	Plasmid 6	
6	*Xba*I	Plasmid 7	2.6
7	No Enzyme	Plasmid 7	
8	*Xba*I	pCWhyg	2.3 and 2,6
9	No Enzyme	pCWhyg	
10	*Xba*I	pR204	4.3
11	No Enzyme	pR204	

Expected *Xba*I fragment sizes:

pCWhyg; 2.3 kb (*hph* cassette) and 2.6 kb.

pR204; 4.3 kb (pGEM-T containing combined flanks)

dotA disruption vector; 2.3 kb (*hph* cassette) and 4.3 kb (pR204).

1 Kb
Plasmid 1 + Xba1
Plasmid 1
Plasmid 6 + Xba1
Plasmid 6
Plasmid 7 + Xba1
Plasmid 7
pCWHyg + Xba1
pCWHyg
pR204 + Xba1
pR204
2.3 kb
4.3 kb

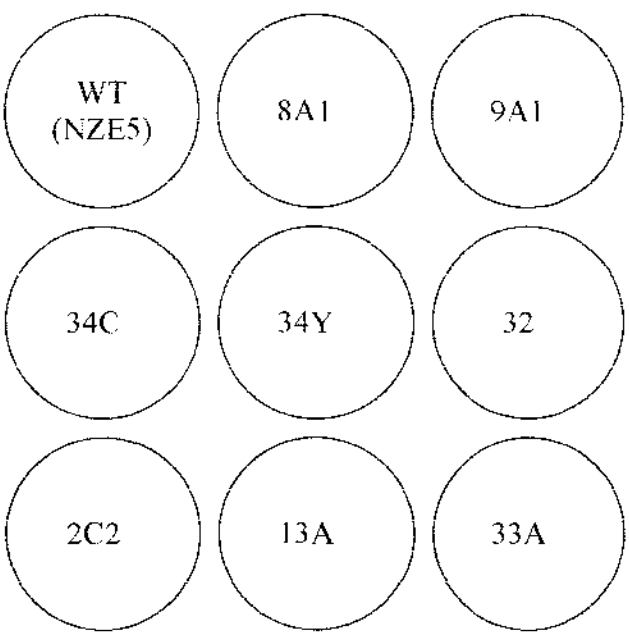
3.3 GENERATION OF DOTA⁻ MUTANTS.

3.3.1 Transformation of NZE5 Protoplasts with *dotA* Disruption Vector.

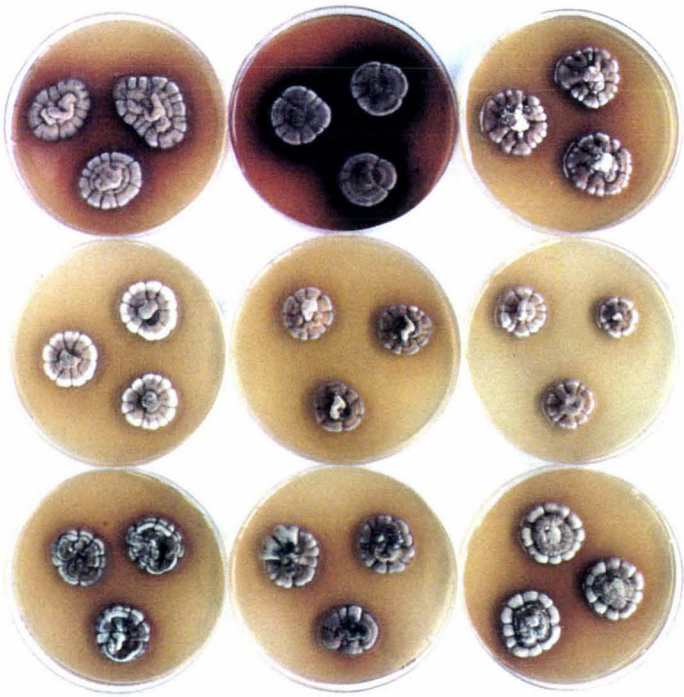
Wild type *D. pini* protoplasts (NZE5 prepared as described in section 2.12.2.1) were transformed with linearised pR208 along with appropriate controls (sections 2.12.2.2) and plated on selective media (70μg/ml HmB in DMsucrose). After 14 days, 37 transformants were subcloned and placed onto fresh DSM + HmB. DSM has been shown to promote spore formation in *D. pini* (Bradshaw, Bidlake *et al.* 1997). 21 days after subcloning onto DSM the transformants were single spore purified (section 2.3.3) onto DSM + HmB media and left to grow for another 21 days to produce colonies that were still discrete. These discrete colonies were used for another round of single spore purification. Some colonies resulting from the second round of single spore purification were phenotypically diverse, with either a) sectors of mycelium that were phenotypically different, b) different morphologies to other colonies derived form the same transformation event or c) differences between colonies produced by separate transformation events (see discussion). In cases where the same transformation event produced different phenotypes, each phenotype was taken and analysed for *dotA* disruption. An example of the range of phenotypes obtained can be seen in figure 3.4. Figure 3.4 shows the excessive pigment produced by colony 8A1. From this observation, it was proposed that colony 8A1 was an over producer of dothistromin.

Figure 3.4 Phenotypic differences in *D. pini* transformed with *dotA* disruption vector.

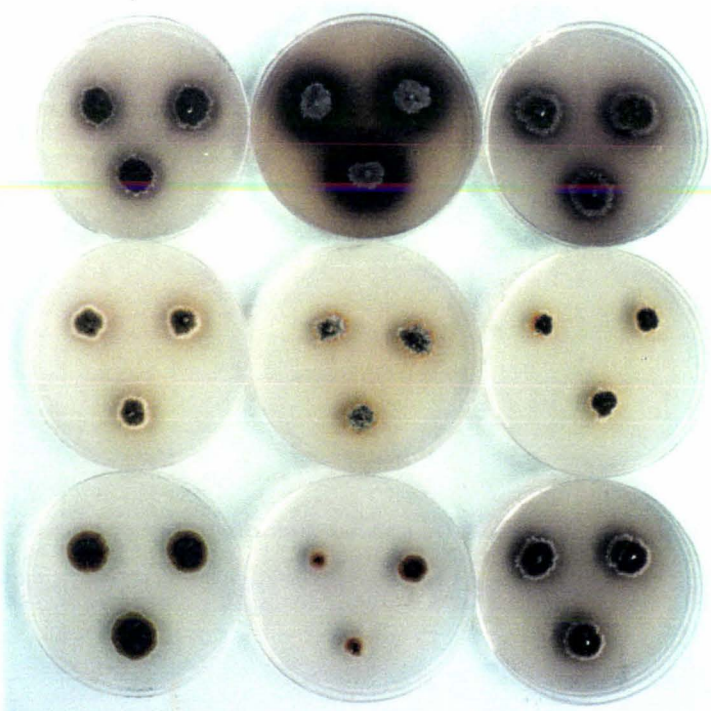
Figure shows morphological differences between transformants and wild type *D. pini*, colonies resulting from different transformation events (each transformation event is denoted by a different number), differences between colonies resulting from the same transformation event (34Y and 34C) and colonies grown on different media (DM and AMM + 2% glucose).



a) Colonies grown on DM for 35 days at 22° C.

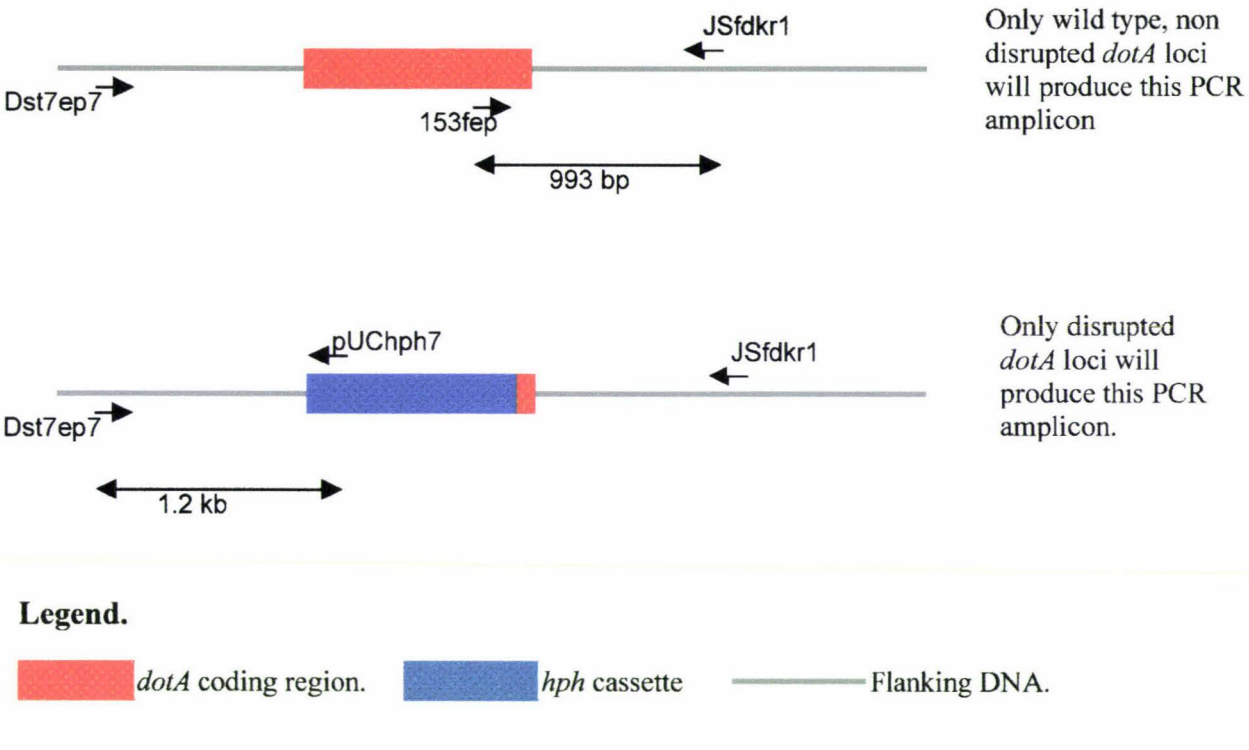


b) Colonies grown on AMM + 2% glucose for 35 days at 22° C.



Colonies were screened for targeted integration of the pR208 vector using two primer sets and the PCR/Microwave method (section 2.13.4). One primer set (pUChph7 and DST7ep7) was designed to give a 1.2 kb PCR product if targeted integration of the pR208 had taken place at the *dotA* locus. The other primer set (JSfdkr1 and 153fep) would give a 993 bp PCR product if the *dotA* locus was still wild type (see Figure 3.5 for primer positions). This was a preliminary screen, as in each case, only one flanking region of the gene was being tested, but it would identify transformants that had not integrated on both flanks.

Figure 3.5 Primer Positions for PCR screen of Putative *dotA* disruptants.



Thirtyseven transformant colonies were screened with PCR (figure 3.5). Thirtyone transformants were identified as having ectopic integration of pR208 (HmB resistant but shown to be wild type at *dotA* locus due to the wild type PCR product amplifying at the 5' flank and lack of amplification at the 3' flank using the primers designed for checking pR208 integration). Six transformants were identified as putative *dotA* disrupted transformants. These putative *dotA* disruptants were listed in table 3.1 along with the results of the PCR screen. Putative disruptants were defined as 'transformants that had produced a PCR product with the primers designed for identification of targeted integration pR208 into *dotA*' or 'a transformant that did not produce a PCR product with the primers designed identify wild type *dotA*'. Two transformants (32 and 34) gave the expected PCR *dotA*$^-$ results with both primer pairs. Transformants producing PCR products for both wild type and disruption primer sets were also included as putative *dotA* disruptants. Two colonies, 8A1 and 13A1 did not produce PCR products with wild type or disruptant primers. It is possible that pigment evident in template restricted Taq polymerase activity. Colonies 8A1 and 13A1 were included in Southern blot to determine integration of pR208. An ectopic transformant (9A1) was included in further experiments as a negative control for targeted integration of pR208. Genomic DNA was isolated from these colonies and wild type NZE5 as described in section 2.5.

The preliminary screen using PCR did not conclusively determine the identity of *dotA* disruptants but did eliminate those that were not disruptants, that is the PCR screen is limited to excluding wild type colonies. Primers designed to check the 5' disruption produced inconsistent results due to difficulties with the PCR amplification. Colonies that were shown to be *dotA* disruptants did not always produce PCR amplicons (results not shown). For this reason the Southern blot analysis of putative disruptants is necessary for conclusive results. To identify *dotA* disruptants, a Southern blot of digested genomic DNA was hybridised with a probe specific for pR208.

Table 3.1 PCR Screen of *D. pini* colonies transformed with R208.

Table lists PCR screen results for wild type PCR products (993 bp from primers 153Fep and JSfdkr1) and integration of the *hph* cassette into the *dotA* 3' flank (pUChph7 and Dst7ep7). PCR products presence is denoted as + and absence as -. See figure 3.5 for details.

Colony	Transformant Group	PCR Screen Results		Special Observations
		Wild type 993 bp PCR product	3' disrupted 1.2 kb PCR product.	
2C2	Putative *dotA* disruptant	+	+	
8A1	Putative *dotA* disruptant	-	-	Appears to be overproducing dothistromin.
9A1	Ectopic	+	-	Colony initially white.
13A	Putative *dotA* disruptant	-	-	
32	Putative *dotA* disruptant	-	+	Colony yellow.
33A1	Putative *dotA* disruptant	+	+	
34Y1	Putative *dotA* disruptant	-	+	Colony yellow
34C1	Putative *dotA* disruptant	-	+	
NZE5	Wild type	+	-	

In addition to those shown here, 30 other transformants were screened using this method and shown to be ectopic.

3.3.2 Identification of *dotA*⁻ mutants.

The targeted disruption of *dotA* was determined by hybridising a Southern blot of genomic DNA. Genomic DNA from each putative *dotA* disruptant, ectopic (9A1) and wild type colony was digested with *Eco*RI and also with *Bam*HI. After gel electrophoresis each digest was transferred to a membrane (section 2.16) and hybridised with a probe designed to distinguish between a wild type *dotA* gene and a disrupted *dotA* gene.

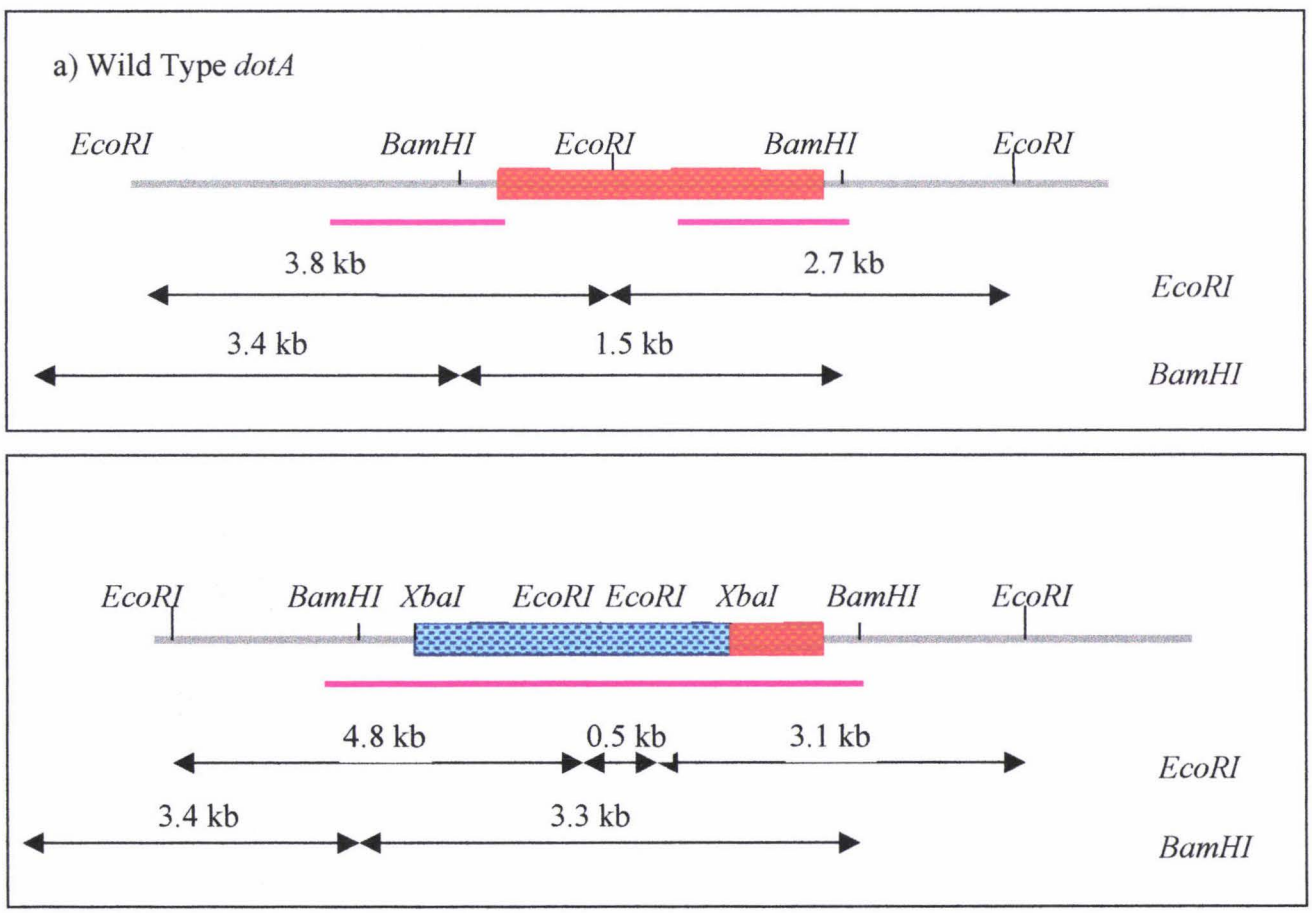
The probe included the *hph* cassette (cut from the pCWhyg vector) and the 1.3 Kb *dotA* flanking regions used in the construction of the disruption vector. The probe was labelled with a random primer DIG labelling system (Roche, section 2.16.2). Expected results of Southern analysis of genomic DNA are described in figure 3.6.

Figure 3.6 Schematic diagram showing expected hybridisation patterns of Southern blot.

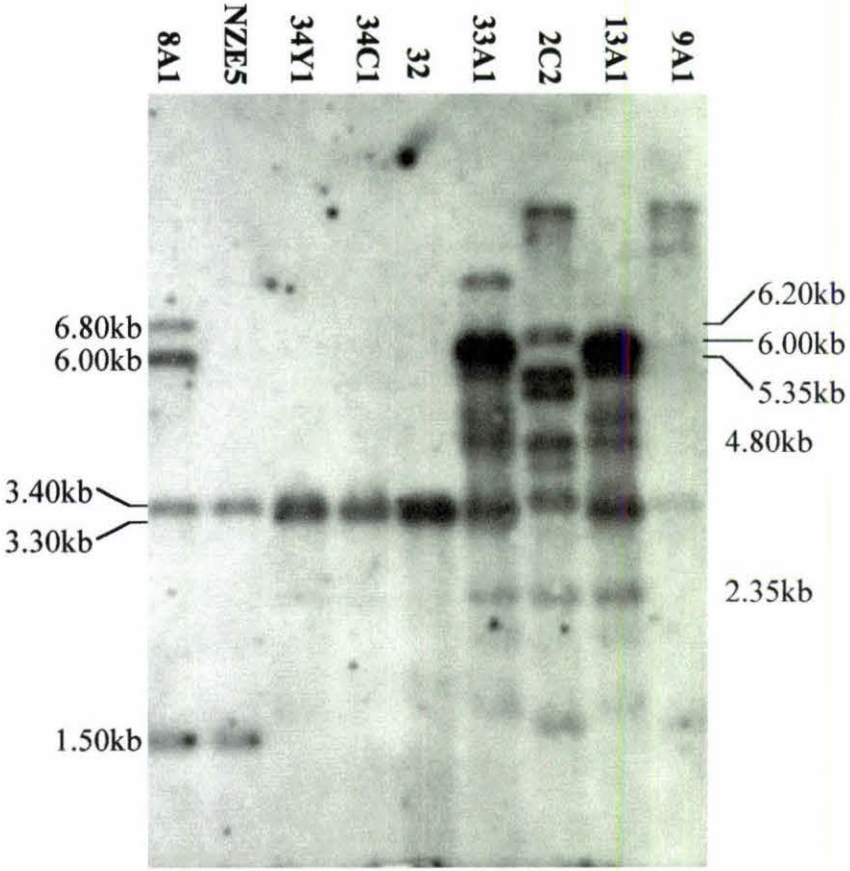
Hybridisation of digested genomic DNA with a labelled probe (*hph* cassette and *dotA* flanking regions) indicates whether the *dotA* locus is wild type or disrupted.

Expected hybridisation fragments.

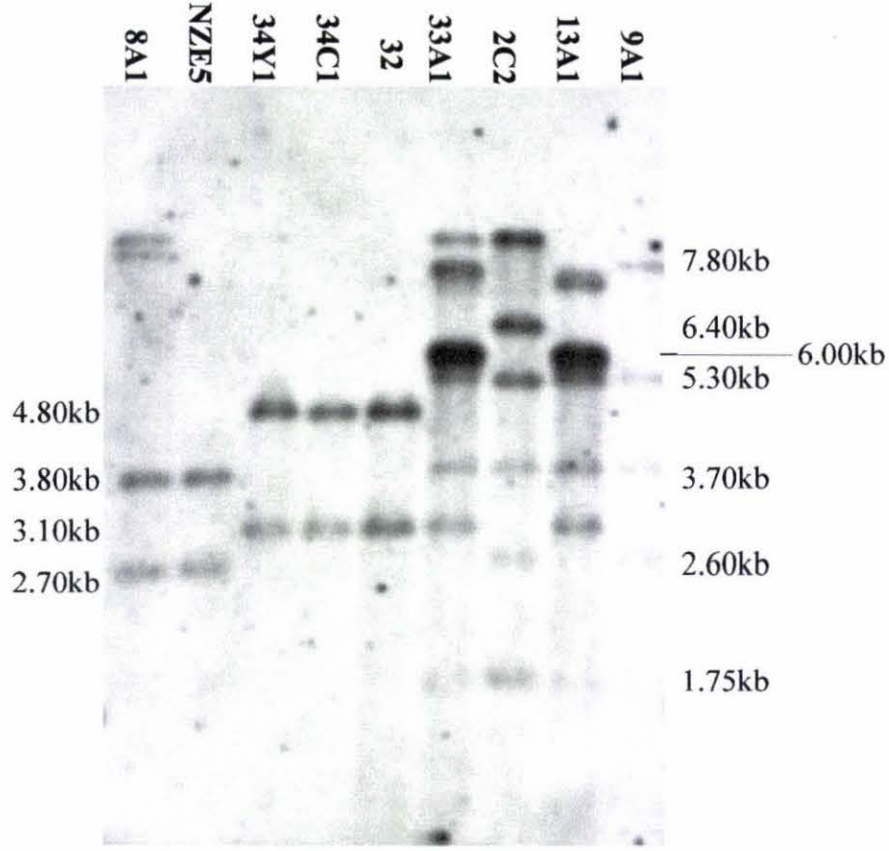
*Eco*RI digests	Wild type: 2.7kb and 3.8kb.
	dotA disruption: 4.8kb, 3.1kb and 0.5kb.
*Bam*HI digests	Wild type: 1.5kb and 3.4kb[1].
	dotA disruption: 3.3kb and 3.4kb.



[1] 3.4kb fragment predicted from previous studies (Monahan, 1998).



Southern Blot of *Bam*HI genomic digests probed with disruption cassette (1.3kb flanking regions and the *hph* cassette).



Southern Blot of *Eco*RI genomic digests probed with disruption cassette (1.3kb flanking regions and the *hph* cassette).

Southern analysis using genomic DNA from each colony determined that colonies 34C1, 34Y1 and 32 had *dotA* disrupted. All three of these colonies had disruption results in both digests, that is a 3.3 kb and 3.4 kb fragment hybridising in the *Bam*HI digest (figure 3.6) and 4.8 kb and 3.1 kb bands hybridising in the *Eco*RI digest (figure 3.6). The colonies 34C1 and 34Y1 originated from the same transformation event, therefore were counted as one transformant. 34Y1 and 34C1 had different morphologies after the first single spore isolation but after further single spore isolations and subcloning, they appeared phenotypically similar. Of the thirty seven transformants analysed, two were *dotA* disruptants (5.4 %). The probe hybridised to the expected fragments in both digests of wild type (NZE5) genomic DNA. Multiple fragments were detected in the other transformants examined. Possibilities as to the origin of these multiple fragments will be examined in the discussion.

3.3.3 ITS sequencing of *dotA*⁻ mutant.

To ensure the *dotA*⁻ mutant was derived from the wild type strain and not a contaminant, sequencing of the ITS1 region was carried out. ITS (internal transcribed sequence) regions are transcribed but non-functional regions of conserved ribosomal DNA (rDNA) in filamentous fungi. Conserved regions of rDNA are interspersed with regions of high variability between related fungal species and strains.

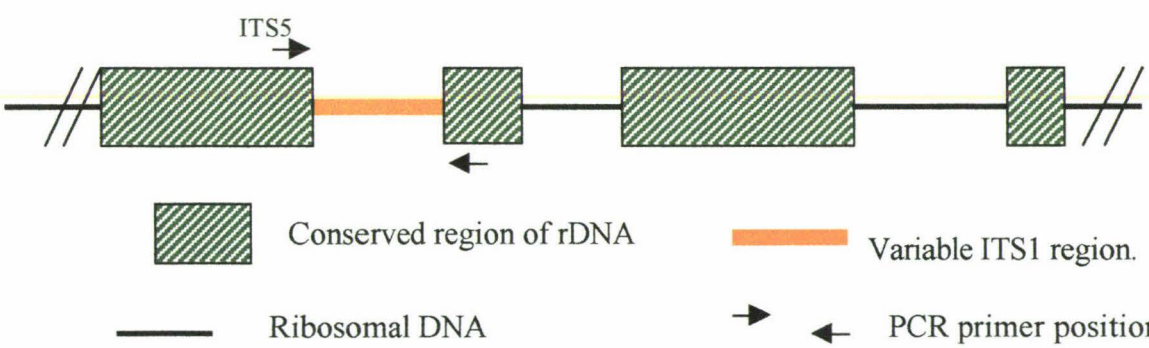


Figure 3.7 Position of ITS1 amplification primers, ITS2 and ITS5 on genomic DNA.

PCR amplification from the conserved regions and sequencing of the product was used to confirm that the *dotA*⁻ mutant and NZE5 are both *D. pini*. Sequences were compared to previously sequenced *D. pini* NZE1 ITS1 region (Bradshaw, Ganley *et al.* 2000) PCR was carried out on genomic DNA using the primers ITS2 and ITS5 (section 2.13.5). Direct sequencing of PCR products showed identical ITS1 region sequence and confirmed 34Y1 was derived from the wild type NZE5 and both 34Y1 and NZE5 are *D. pini* (by comparison to NZE1 sequence, Figure 3.8)

```
                      10         20         30         40         50
                       |          |          |          |          |
34Y1 ITS1        GTTTAAATTTACTTTAAGACTCCGACGCAAAGATGCAGTGTTTGATGACCTCCGGGGGC
NZE5 ITS1        GTTTAAATTTACTTTAAGACTCCGACGCAAAGATGCAGTGTTTGATGACCTCCGGGGGC
NZE1 ITS1        GTTTAAATTTACTTTAAGACTCCGACGCAAAGATGCAGTGTTTGATGACCTCCGGGGGC

                 60         70         80         90         100        110
                 |          |          |          |          |          |
34Y1 ITS1        GCCGTCGCCGAAACGGCGGGGTCGCCCCCGAAGCAACAGAGTTGGTTCACAAAGGGTTG
NZE5.ITS1        GCCGTCGCCGAAACGGCGGGGTCGCCCCCGAAGCAACAGAGTTGGTTCACAAAGGGTTG
NZE1 ITS1        GCCGTCGCCGAAACGGCGGGGTCGCCCCCGAAGCAACAGAGTTGGTTCACAAAGGGTTG

                 120        130        140
                 |          |          |
34Y1 ITS1        GAGGTCGGGCTTTCGCCCTCACTCAG
NZE5.ITS1        GAGGTCGGGCTTTCGCCCTCACTCAG
NZE1 ITS1        GAGGTCGGGCTTTCGCCCTCACTCAG
```

Figure 3.8 Multiple alignment of ITS1 regions.

Figure compares ITS1 nucleotide sequences from *dotA*⁻ mutant 34Y1, wild type colony NZE5 and *D. pini* wild type strain NZE1.

3.4 DETERMINATION OF *DOTA* FUNCTION.

The successful disruption of the putative dothistromin biosynthetic gene *dotA* allows analysis into the function of that gene. Gene disruption is a common technique for determining gene function in filamentous fungi. The gene *dotA* is thought to act on the dothistromin biosynthetic pathway. A number of pathways for the biosynthesis of dothistromin are possible, and although compounds thought to be intermediates on the pathway have been found in *D. pini* (Figure 1.2) the biosynthetic pathway is presently unknown.

The *dotA* homolog in *A. parasiticus (ver1A)* has been disrupted. The resulting *ver1A*$^-$ mutants did not produce AF but accumulated a fluorescent yellow pigment in the mycelium the major component of which was versicolorin A (Skory, Chang *et al.* 1992). It is hypothesised that *dotA* catalyses the reduction of versicolorin B. Versicolorin B and dothistromin both lack a double bond present in versicolorin A and AF. Versicolorin B has been detected in *D. pini* in previous studies (Section 1.4.1, Figure 1.2) and is thought to be an intermediate on the dothistromin biosynthetic pathway. Versicolorin B is also an intermediate on the AF and ST pathways, and this, coupled with the presence of genes homologous to AF biosynthetic genes led to the proposal that under certain conditions, *D. pini* could produce AF. AF production was tested under different conditions, none of them producing AF (Ganley 2000). Accumulation of intermediates in the *dotA*$^-$ mutants will indicate at which step *dotA* functions.

The *dotA* mutants will be examined for dothistromin production, accumulation of putative dothistromin intermediates and growth rates to investigate the function of *dotA*.

3.4.1 Effect of *dotA* disruption on the growth rate of *D. pini.*

Disruption of *dotA* was not expected to hinder growth of the fungus due to its putative role on the pathway of a secondary metabolite. The radial growth rates of the mutant cultures (32 and 34Y1) were compared to wild type culture (NZE5) and the putative overproducing strain (8A1). Table 3.2 summarises the data from two types of media,

AMM+2% glucose and DM. These data were also used to determine the length of time, relative to wild type, the mutant cultures needed to be grown before they could be harvested for dothistromin ELISA tests.

Colonies were grown on solid AMM + 2% glucose (2.2.10) and DM (2.2.4) for 35 days and measured at 4, 7, 10, 14, 18, 21, 28 and 35 days (technical assistance of R. Bradshaw is acknowledged).

Table 3. 2 Growth Rates of *dotA* Mutants.

Mean radial growth rates (mm) on AMM + 2% glucose (n=6)

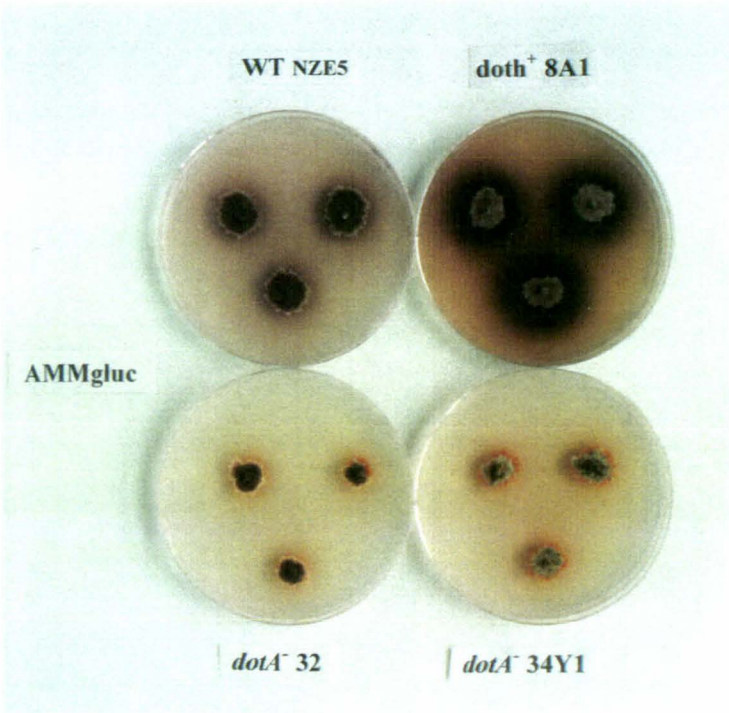
	4 d	7 d	10 d	14 d	18 d	21 d	28 d	35 d
NZE5	2.6 ± 0.6	3.6 ± 0.5	5.9 ± 0.2	8.0 ± 0.6	9.5 ± 0.5	11.2 ± 0.4	13.5 ± 0.8	15.5 ± 0.8
8A1	2.3 ± 0.4	3.9 ± 0.8	5.0 ± 0.7	7.3 ± 0.4	8.9 ± 0.7	10.7 ± 0.5	12.0 ± 0.0	14.0 ± 0.6
32	2.0 ± 0.0	2.5 ± 0.5	3.8 ± 0.5	5.7 ± 0.5	7.1 ± 0.7	7.3 ± 0.5	9.5 ± 0.8	11.0 ± 0.9
34C1	2.5 ± 0.0	2.9 ± 0.5	4.8 ± 0.4	6.0 ± 0.0	7.5 ± 0.5	8.5 ± 0.6	10.7 ± 0.8	12.7 ± 0.8
34Y1	2.5 ± 0.3	3.5 ± 0.4	4.2 ± 0.4	5.8 ± 0.4	8.0 ± 1.4	8.4 ± 0.8	10.7 ± 1.0	13.0 ± 0.6

Mean radial growth rates (mm) on DM (n=6)

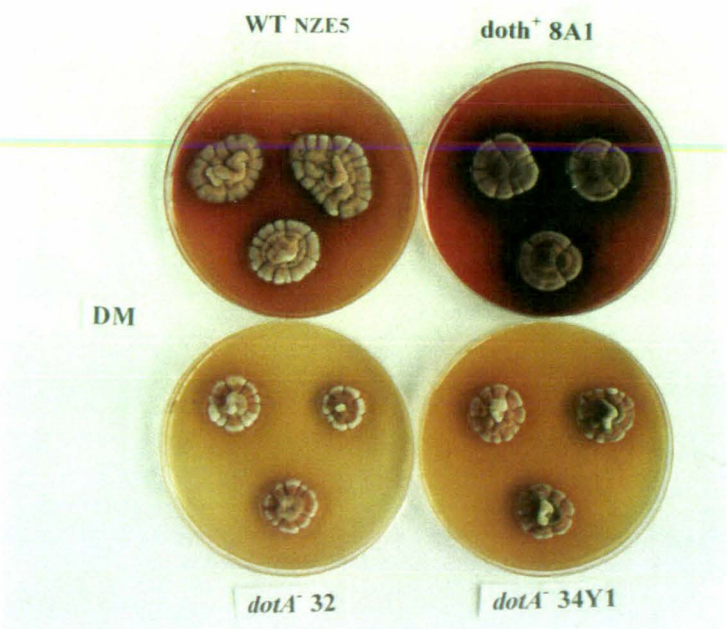
	4 days	7 days	10 days	14 days	18 days	21 days	28 days	35 days
NZE5	4.8 ± 0.5	6.1 ± 0.7	8.2 ± 0.5	11.0 ± 0.6	13.3 ± 0.8	15.7 ± 1.2	20.0 ± 0.9	23.5 ± 1.4
8A1	3.4 ± 0.2	4.8 ± 0.3	7.8 ± 0.3	10.7 ± 0.8	13.5 ± 0.5	15.2 ± 0.5	18.7 ± 0.5	20.2 ± 0.8
32	2.2 ± 0.3	2.3 ± 0.4	3.1 ± 0.7	5.0 ± 0.7	7.6 ± 0.5	9.6 ± 0.5	13.8 ± 0.5	18.0 ± 1.6
34C1 .	3.0 ± 0.3	4.9 ± 0.4	6.5 ± 0.4	8.8 ± 0.4	11.9 ± 0.4	13.6 ± 0.5	18.3 ± 0.5	22.2 ± 0.75
34Y1	3.1 ± 0.4	4.8 ± 0.3	6.8 ± 0.3	9.8 ± 0.4	12.5 ± 0.6	13.7 ± 1.2	17.5 ± 2.3	20.8 ± 2.7

Figure 3.9 **Morphological Comparison of Wild Type *D. pini* to *dotA*⁻ Mutants and Putative Dothistromin Overproducing Strain 8A1.**

a) AMM + 2% glucose



b) Dothistromin Media



Colony 32 appears to be growing at a slower rate than the other strains on both media but it is not too slow to continue with the trials with this strain. Cultivation of the mutant strains on the colourless AMM+2% glucose media enabled the yellow pigment being released into the media to be easily seen. Dothistromin production was also easily seen from both NZE5 and 8A1. Dothistromin appeared to be black on the AMM+2% glucose plates compared with its dark brown colour seen on the DM plates (see figure 3.9).

3.4.2 Competitive Enzyme-Linked Immunosorbant Assay (ELISA) To Quantify Dothistromin Production.

ELISA assays were developed at Hort Research in order to rapidly quantify dothistromin and examine it's role in fungal/plant interactions (Jones, Harvey *et al.* 1993). ELISA assays have been used in our lab to quantify the dothistromin production of isolates collected throughout New Zealand and compare them to dothistromin production by foreign isolates (Ganley 2000). In this project, ELISAs will be used to determine whether the *dotA* mutants are impaired in dothistromin production compared to the wild type.

The ELISA for dothstromin is competitive. Anti-dothistromin antibodies are added to sample. If dothistromin is present, the antibodies bind to it. The sample/antibody mix is then placed in a plate coated in dothistromin. If the antibodies are already bound to dothistromin from the sample, they will not be bound to the plate and be washed away before the detection of their peroxidase label. If there is no dothistromin in the sample, the antibodies are free to bind to the dothistromin coated plate and will be detected. The Peroxidase acts on the substrate and converts it so it can be detected by absorbance measured at 492 nm. The higher the absorbance the lower the levels of dothistromin in the sample. The mean of eight samples containing no dothistromin (B_0) was used as a baseline measurement. The % inhibition was calculated for each sample by subtracting the sample absorbance (B) from B_0 and dividing B_0-B by B_0 (data in appendix 4). % inhibition is proportional to dothistromin concentration. A standard curve was constructed using dothistromin samples ranging from 0.75 ng/mL to 1 μg/mL (figure 3.10).

Due to the non-linearity of the standard curve as it approached 0 the lowest accurate % inhibition reading is 17 (22.44 ng/mL). Dothistromin concentrations below this value were 'too low' to calculate from this curve. These concentrations are listed as less than 22.44 ng/mL with dilutions also taken into account.

Triplicate samples were prepared from strains 34Y1, 32, wild type NZE5 and putative dothistromin overproducer 8A1 from two time points, 7 and 9 days. Dry weights of mycelia were measured and used to determine dothistromin production per weight. The growth culture supernatant's were used in ELISA as described in section 2.15. Data calculations are shown in appendix 4. Previous studies have diluted wild type and overproducing strains 10 fold in order for the samples to be in the linear portion of the standard curve. For this reason samples from strains NZE5 and 8A1were diluted 10 fold.

The concentrations of dothistromin in the mutants and some wild type strains were too low to calculate, and above 17, % inhibition is linearly related to dothistromin concentration. Comparisons between the % inhibition values have been made to investigate the statistical significance of differences between mutants and wild type strains.

dotA disruption mutants 32 and 34 are not producing dothistromin to the same extent as the wild type or strain 8. In samples where the % inhibition was too low to be read off the standard curve, the lowest accurate % inhibition was taken to be 17 giving in the mutants a maximum dothistromin concentration of 22.44 ng/mL. The wild type samples that were too low to read, were diluted 10 times more than the mutant strains. These data give us an approximate range in which to compare wild type to mutant strain dothistromin production. The highest possible dothistromin production by mutants (22.44 ng/mL) is 10 times lower than a wild type strain with the same % inhibition and 43 times lower than the highest wild type producer of dothistromin. This gives us a qualitative relationship of mutants producing 10 - 43 times less dothistromin than the wild type strain. These data indicate that the disruption of *dotA* decreases dothistromin production of *D. pini*. Table 3.4 lists the data used for the results.

Figure 3.11 shows a clear decrease in dothistromin concentration in mutant strains 32 and 34 when compared to wild type strain and strain 8A1. Because % inhibition for both mutants is less than the accuracy value of 17, statistical comparisons of dothistromin production between the two mutant strains is not possible.

Comparisons between the absorbance of samples from colonies 32 and 34 to the eight Bo (no dothistromin) controls used in the ELISA assay shows the mutants strains are very close to this level (figure 3.12). The variation of absorbance readings from identical Bo samples shows the inaccuracy of the assay as dothistromin levels approach or reach 0. Because of the variations within the Bo absorbances, the inaccuracy of the standard curve and the closeness of some of the mutant readings to the 0 dothistromin standards, it cannot be confirmed that either mutant strain is producing dothistromin. This comparison is qualitative so statistical analysis is not possible.

Using highest estimates for wild type strains with an inhibition of less than 17%, the amount of dothistromin secreted into the media by strains NZE5 and 8A1 from different days was compared.

Table 3.3 *t* testing between mean dothistromin production by strains NZE5 and 8A1.

Sample	$\overline{x}$ (Mean)	*s* (Standard deviation)	n Sample size	*t*	df	p	*Ho*
NZE5 9 days (μg dothistromin/mL)	0.29	0.07	3	2.7	3	0.03	Rejected
8A1 9 days (μg dothistromin/mL)	0.51	0.12	3				
NZE5 9 days (μg dothistromin/mg mycelium)	2.03	0.33	3	1.37	3	0.14	Accepted
8A1 9 days (μg dothistromin/mg mycelium)	2.56	0.59	3				
NZE5 7 days (μg dothistromin/mL)	0.48	0.43	3	0.58	2	0.5	Accepted
8A1 7 days (μg dothistromin/mL)	0.34	0.12	3				
NZE5 7 days (μg dothistromin/mg mycelium)	1.52	1.53	3	0.15	2	0.43	Accepted
8A1 7 days (μg dothistromin/mg mycelium)	1.66	0.51	3				

Ho, the null hypothesis is that there is no difference between the sample mean of 8A1 and NZE5.

t testing of the difference between the two means showed that with only 3 samples, the difference between dothistromin production at nine days is significant (at 95% significance) and therefore there is a difference in media dothistromin concentration between the two strains at 9 days. *t* testing to compare dothistromin production standardised to dry weight of mycelia show that strain 8A1 does not produce more dothistromin per mg dry weight mycelia than the wild type. Calculations can be found in appendix 5 and table 3.3. Means calculated for the wild type strain used estimates, therefore results described here are not conclusive.

Figure 3.10 Standard Curve of Dothistromin Concentration.

Standard curve was constructed from the % inhibition of known dothistromin concentrations (data in appendix 4). The standard curve loses accuracy as it approaches 0 and the lowest % inhibition reading taken from it was from 17.

Figure 3.10 **Standard Curve Showing The % Inhibition Of Standards With Known Dothistromin Concentration.**

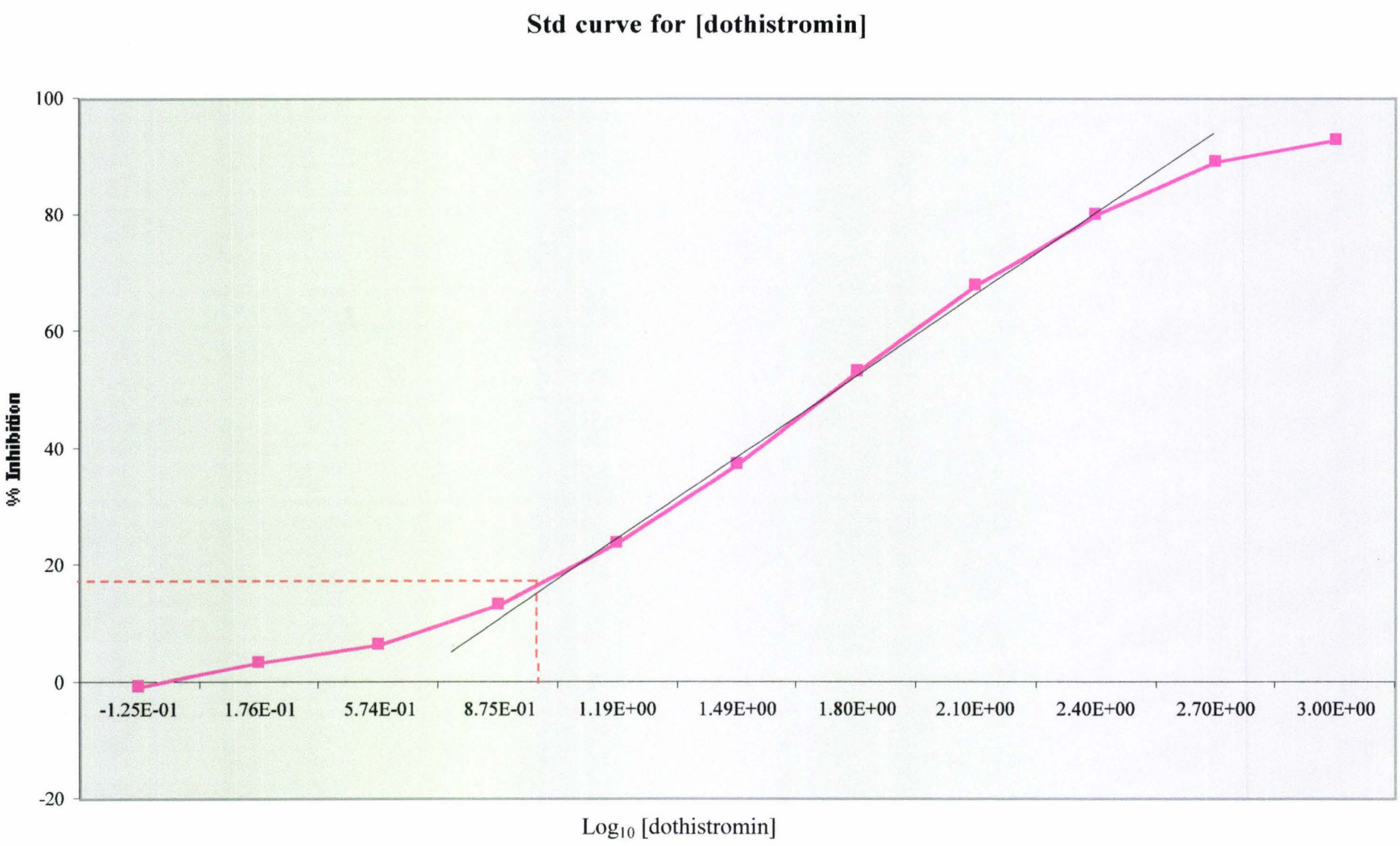


Figure 3.11 Comparison of Average % Inhibitions.

The average % inhibition for each strain (n=3) is plotted with the range of the standard deviation. Standard deviations of the mutant strains (32 and 34) were too small to be plotted at this scale.

Table 3.4 Data Used To Calculate Dothistromin Concentration In Mutant And Wild Type Strains.

Sample	Dilution Factor	% Inhibition	Mean % Inhibition	[dothistromin] (μg/ml)	Mean [dothistromin] (μg/ml)	Mean μg dothistromin/ mg mycelium
WTA1	10	18.86		0.25		
WTB1	10	12.42	26.11 ± 18.95	≤0.22[A]	0.48 ± 0.43[B]	1.52 ± 1.53
WTC1	10	47.73		0.98		
WTA2	10	9.74		≤0.22		
WTB2	10	26.91	19.60 ± 8.90	0.362	0.29 ± 0.07[B]	2.03 ± 0.33
WTC2	10	22.08		0.289		
32A1	1	3.73		≤0.02		
32B1	1	6.20	5.70 ± 1.77	≤0.02		
32C1	1	7.16		≤0.02		
32A2	1	2.12		≤0.02		
32B2	1	1.91	3.84 ± 3.16	≤0.02		
32C2	1	7.49		≤0.02		
34A1	1	14.25		≤0.02		
34B1	1	9.63	7.27 ± 8.41	≤0.02		
34C1	1	-2.07		≤0.02		
34A2	1	7.59		≤0.02		
34B2	1	5.23	3.69 ± 4.85	≤0.02		
34C2	1	-1.74		≤0.02		
8A1	10	31.63		0.47		
8B1	10	23.05	24.12 ± 7.04	0.30	0.34 ± 0.12	1.66 ± 0.51
8C1	10	17.68		0.24		
8A2	10	30.45		0.43		
8B2	10	31.53	33.74 ± 4.80	0.46	0.51 ± 0.12	2.56 ± 0.59
8C2	10	39.25		0.65		

WT = Wild type; 8 = Strain 8A1; 34 = 34Y1; A, B or C indicates flask sample replicates taken from; 1= 7 days; 2 = 9 days. Eg: 8B2 = Strain 8A1, 9 days, second inoculation.

[A] % Inhibition values below 17 are inaccurate and therefore are unable to be used to calculate dothistromin concentration. The [dothistromin] at % inhibition is 22.44 ng/ml. Samples with % inhibition values lower than 17 are said to contain ≤ 22.44 ng/ml dothistromin.

[B] Means are estimates, as highest estimates (see footnote A) have been used in calculation.

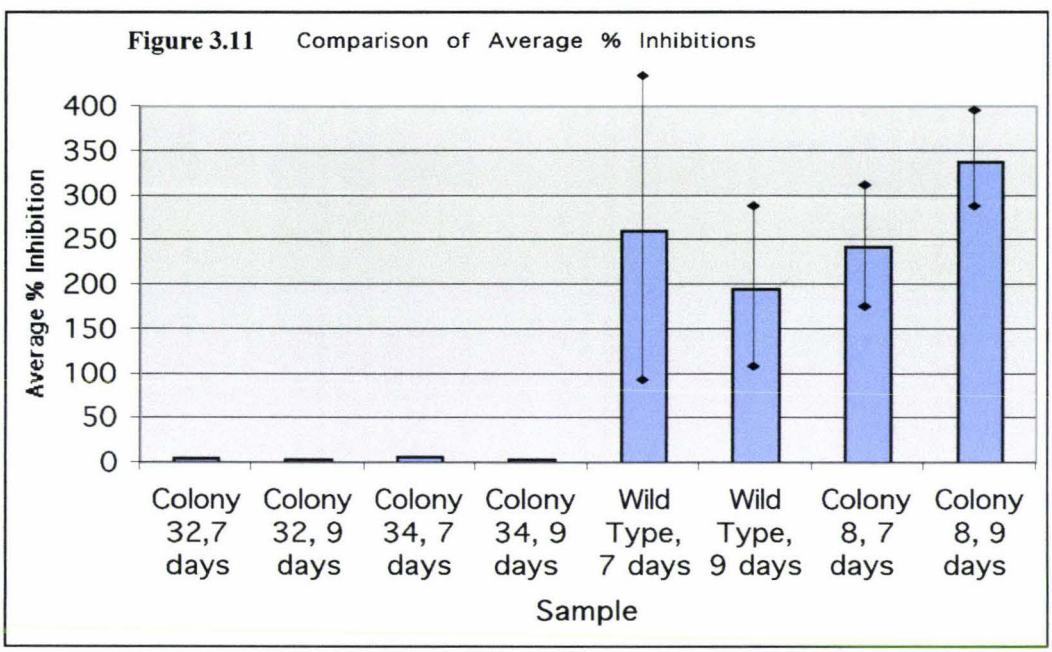
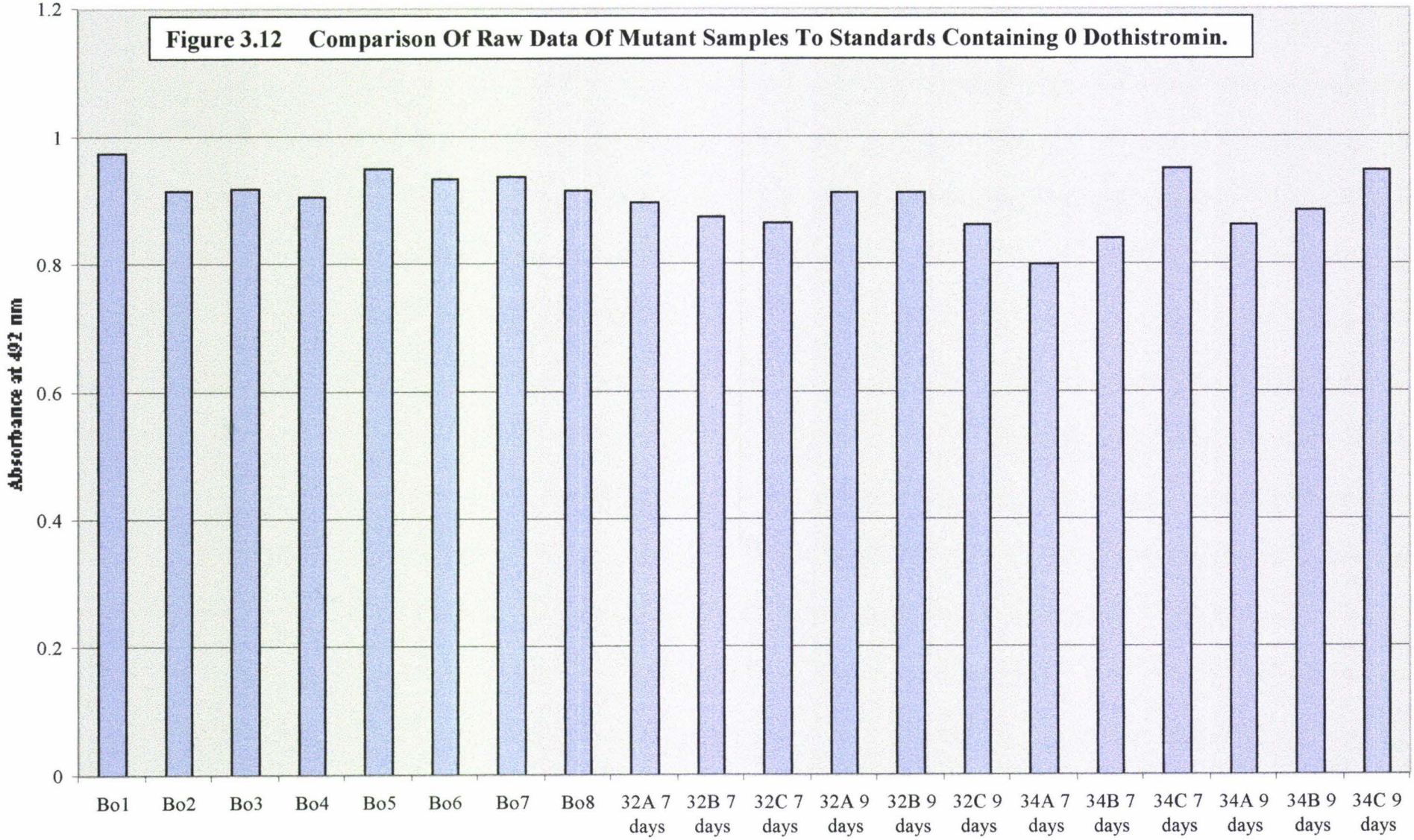


Figure 3.11 Comparison of Average % Inhibitions
Average % Inhibition
400
350
300
250
200
150
100
50
0
Colony 32,7 days
Colony 32, 9 days
Colony 34, 7 days
Colony 34, 9 days
Wild Type, 7 days
Wild Type, 9 days
Colony 8, 7 days
Colony 8, 9 days
Sample

Figure 3.12 Chart to show variation in the ELISA readings of 0 dothistromin compared to ELISA readings of mutant strain 34Y1 and 32.

Bo samples contain no dothistromin. Absorbance measured the amount of labelled dothistromin antibodies able to bind to the plate due to lack of dothistromin in the sample. Absorbance at 492 nm is inversely proportional to dothistromin in sample.

Figure 3.12 Comparison Of Raw Data Of Mutant Samples To Standards Containing 0 Dothistromin.



3.4.3 Analysis of wild type and mutant strains for accumulation of AF intermediates.

The phenotype of the mutant colonies showed the accumulation of a yellow pigment in the mycelia and in the media. When compared to the wild type colony or the putative dothistromin overproducing colony (8A1), the difference in phenotype was quite marked (see figure 3.9).

The yellow colouration of the *dotA*$^{-}$ mutant colonies and the surrounding media, was an encouraging sign that the mutants could be accumulating a yellow intermediate rather than producing dark brown dothistromin.

Secondary metabolites were extracted from 800 mL cultures grown with shaking for 7 and 9 days at 22°C. Samples were taken from mutant strain 34Y1 and wild type NZE5(section 2.17) and condensed before being sent to the Southern Regional Research Centre, United States Department of Agriculture, New Orleans, U.S.A. for characterisation of the pigments by Thin Layer Chromatography (TLC) (section 2.18) and Mass Spectometry (MS). Early TLC results indicate the presence of versicolorin A in mutant extract.

3.5 DISCUSSION

3.5.1 Generation, Transformation and Regeneration of *D. Pini* Protoplasts.

The transformation frequencies achieved using the new enzyme glucanex and NZE5 are comparable those achieved in previous studies that used novozyme and NZE1 (Bidlake 1996). The results also confirm the protocol to be successful in the transformation of *D. pini* protoplasts and that pCWhyg is able to confer HmB resistance on *D. pini*.

After transformation smaller abortive transformants were evident on HmB plates. Abortive transformants have been transformed with a resistance plasmid that has not integrated into the genome. The antibiotic resistance gene is expressed, but because the plasmid is not integrated, it is not replicated and is only able to confer transient resistance to the colony, resulting in a small antibiotic resistant colony. This phenomenon has previously been observed in *D. pini* (Bradshaw, Bidlake *et al.* 1997) and in the transformation of other fungi ((Rikkerink, Solon *et al.* 1994); (Wang, Kim *et al.* 1999); (Cooley, Shaw *et al.* 1988)) .

3.5.2 Construction of *dotA* disruption vector.

It is generally accepted that the longer the flanking regions, the greater the chance of targeted disruption occurring via homologous recombination in fungi. Disruption of the *dotA* homolog in *A. parasticus* was achieved using a vector with flanking regions of 2.2 kb (Liang, Skory *et al.* 1996). A study looking at the frequency of targeted disruption with respect to the length of homologous sequence in the disruption vector (Shiotani and Tsuge 1995) found that in the filamentous fungi *Alternaria alternata*, longer homologous regions increased targeted recombination when the fungus was transformed with the circular vector but made no difference if the vector was linearised prior to transformation. Frequency of targeted integration of circular vectors was also found to be dependant on length of homology in *A. nidulans* (Bird and Bradshaw 1997). Constraints such as the proximity of adjacent genes and primer hybridisation sites recommended by the primer design software

(section 3.2.1), meant the flanking regions in the *dotA* disruption vector were only 600 to 700 bp long and shorter than desired. However, the vector was linearised prior to transformation in order to increase the likelihood of targeted disruption.

Three *E. coli* colonies screened after preliminary PCR screens indicated they could contain pGEM-T with the desired flanking region/*hph* cassette ligation product. Two colonies (1 & 7) produced an unexpected 2.6 kb *Xba*I DNA fragment. 2.6kb is the size of pCWhyg without the *hph* cassette present. It is possible that when the 2.3kb *hph* cassette was gel extracted from pCWhyg, the *hph* cassette gel slice was contaminated with nearby 2.6kb DNA fragments which then religated and were transformed into *E. coli* colonies 1 and 7. When digested and examined with gel electrophoresis, these were evident as small plasmids and 2.6kb *Xba*I fragments

3.5.3 Analysis of pR208 transformants and confirmation of *dotA*⁻ mutants.

Laboratory stocks of *D. pini* are phenotypically diverse in their appearance and in dothistromin production (Bradshaw, Ganley *et al.* 2000) even in clonal colonies. Morphological variability in plant pathogens is common. The removal of the pathogen from its natural environment is expected to cause the morphological variation. Genomic instability could also contribute to phenotypic variation and can be caused by chromosome rearrangements, mycoviral infection and/or transposable elements (Daboussi 1997).

The transformants were more diverse in their appearance than wild type stocks (figure 3.3). The phenotype of the purified *dotA* disruptants were very similar to each other, while the ectopic transformants were phenotypically varied. Ectopic integration events (via non-homologous recombination) are common in fungal transformation systems. It is ectopic integration into seemingly random loci that allows plasmids such as pAN7-1 and pCWhyg to confer resistance to *D. pini.* The different phenotypes between transformants can be attributed to the ectopic integration of pR208. The variations are thought to be caused by integration of the disruption vector at many different loci in the genome or homologous recombination into one flanking region of the gene. Ectopic integration can affect activity

of genes by disrupting genes not targeted or integrating close to genes and interfering with gene expression.

The range of different phenotypes between colonies derived from the same transformation event could be due to the formation of heterokaryons. Transformation facilitated by PEG, clumps the protoplasts together and traps DNA in between the protoplasts increasing the chance of transformation (Fincham 1989). PEG also facilitates protoplast fusion (Cooley and Caten 1993). The fungal mycoplasm can contain more than one nucleus. When two protoplasts fuse, their nuclei can stay discrete forming a heterokaryon. Single spore purification is carried out in order to decrease the chance of heterokaryons being produced. It is postulated the colonies produced from the same original transformant are morphologically different due to heterokaryon formation and separation of the nuclei into different colonies in the single spore isolation.

Phenotypic variations were seen in early isolations of the *dotA* mutants 34C1 and 34Y1. The first single spore isolation of transformant 34 resulted in 4 phenotypic variations. On the first single spore isolation plate the colony 34C1 was dark green and the colony 34Y1 was bright yellow but for both, preliminary PCR screening and later the Southern blot confirmed *dotA* disruption. Further rounds of single spore isolation and subcloning of these two colonies indicated both had the same yellow phenotype. This suggests the initial colony 34 was a heterokaryon and one nuclear type was diluted out in the rounds of single spore purification and sub cloning.

Hybridised fragments seen in 13A and 33A1 are similar. Both have the presence of 3.8kb and 3.05kb hybridised fragments in the *Eco*RI genomic digest and 3.3kb hybridised fragment in the *Bam*HI digest. These bands are equivalent to hybridised bands seen in the *dotA* disruptants and suggest 3' integration of the *dotA* disruption vector in 13A and 33A1. 13A also had a 1.5kb hybridised band in the *Bam*HI digest, indicating the presence of the *dotA* 5' wild type region. These could have arisen through a crossover event at one flank and ectopic integration of the rest of the *hph* cassette without disruption of *dotA* Preliminary PCR screen results do not support this hypothesis for colony 13A but support 3' integration of the disruption vector in 33A1 (Table 3.1). Preliminary PCR resulted in

products for both wild type and disruptant primer sets in colony 2C2, but the presence of wild type hybridising fragments in both digests confirm it is not a disruptant.

The Southern blot shows evidence that the strain 8A1 has a simpler integration event than the other ectopic colonies and has a wild type *dotA* gene. The preliminary PCR screen for the strain 8A1 did not produce any products, however the PCR template for this strain had high levels of pigment which could have inhibited *Taq* polymerase action. As the Southern blot indicates only two bands of hybridisation apart from the wild type *dotA* bands in both digests, it is hypothesised that the *hph* cassette has integrated into one or two places in the genome.

3.5.4 Analysis of *dotA*⁻ mutants.

The results of the Southern blot confirm that the *dotA* gene has been successfully disrupted in *D. pini* strains 32, 34Y1 and 34C1 with the 2.3 kb *hph* cassette. ELISA assays and TLC analysis of strain 34 suggest that disruption of *dotA* terminates or severely restricts dothistromin production and leads to accumulation of versicolorin A therefore it can be proposed that the *D. pini dotA* gene is involved with dothistromin biosynthesis and functions in the conversion of versicolorin A. It was expected that versicolorin B would be accumulated due to its previous detection in *D. pini* and the similarity to the structure of dothistromin.

Detection of versicolorin A by TLC in the mutant strain 34 (personal communication, D. Bhatnagar) indicates how *D. pini* synthesises dothistromin. Three possible dothistromin pathways have been suggested (M. Chick, personal communication) only one of which contains versicolorin A. One pathway suggested by Chick involves the desaturation of versicolorin B to versicolorin A and reduction of the double bond (found in Versicolorin A but not in either versicolorin B or dothistromin) at the end of the pathway (Figure 3.13). The detection of 6-deoxyversicolorin A IX (indicated on fig 3.13) (Danks and Hodges 1974) provides further support to this hypothesis. The desaturation of versicolorin B to versicolorin A on the dothistromin biosynthetic pathway would make the bisfuran moiety

more reactive for easy placement of the hydroxyl group needed to produce dothistromin (personal communication K. Ehrlich).

Disruption of the *dotA* homolog *ver1A* in *A. parasiticus* led to accumulation of a yellow pigment in the mycelia (AF intermediate versicolorin A) and lack of AF production (Skory, Chang *et al.* 1992). *A. parasiticus* also contains *ver1B,* a non-functional homolog of *ver1A* (95% identical). When the AF$^-$ mutant with *ver1A* disrupted was transformed with a functional copy of *ver1A*, AF production continued and the mutant no longer accumulated versicolorin A. This confirmed that *ver1A* is the only functional copy in *A. parasiticus* (Liang, Skory *et al.* 1996). The gene *ver1B* is not functional due to the presence of a premature stop codon. Although the amino acid identity between *D. pini* homologs *dotA* and *phn1* is only 68%, the homology is enough to suggest functional complementation is possible especially as *phn1* does not contain a premature stop codon.

If further conclusive studies show a small amount of dothistromin is still produced in the mutant, it is possible that *phn1* is catalysing the reaction usually catalysed by *dotA*. In order to quantify dothistromin production in the mutant strains, secondary metabolites should be concentrated by solvent extraction and ELISA assays repeated.

3.5.5 Analysis of putative dothistromin overproducer 8A1.

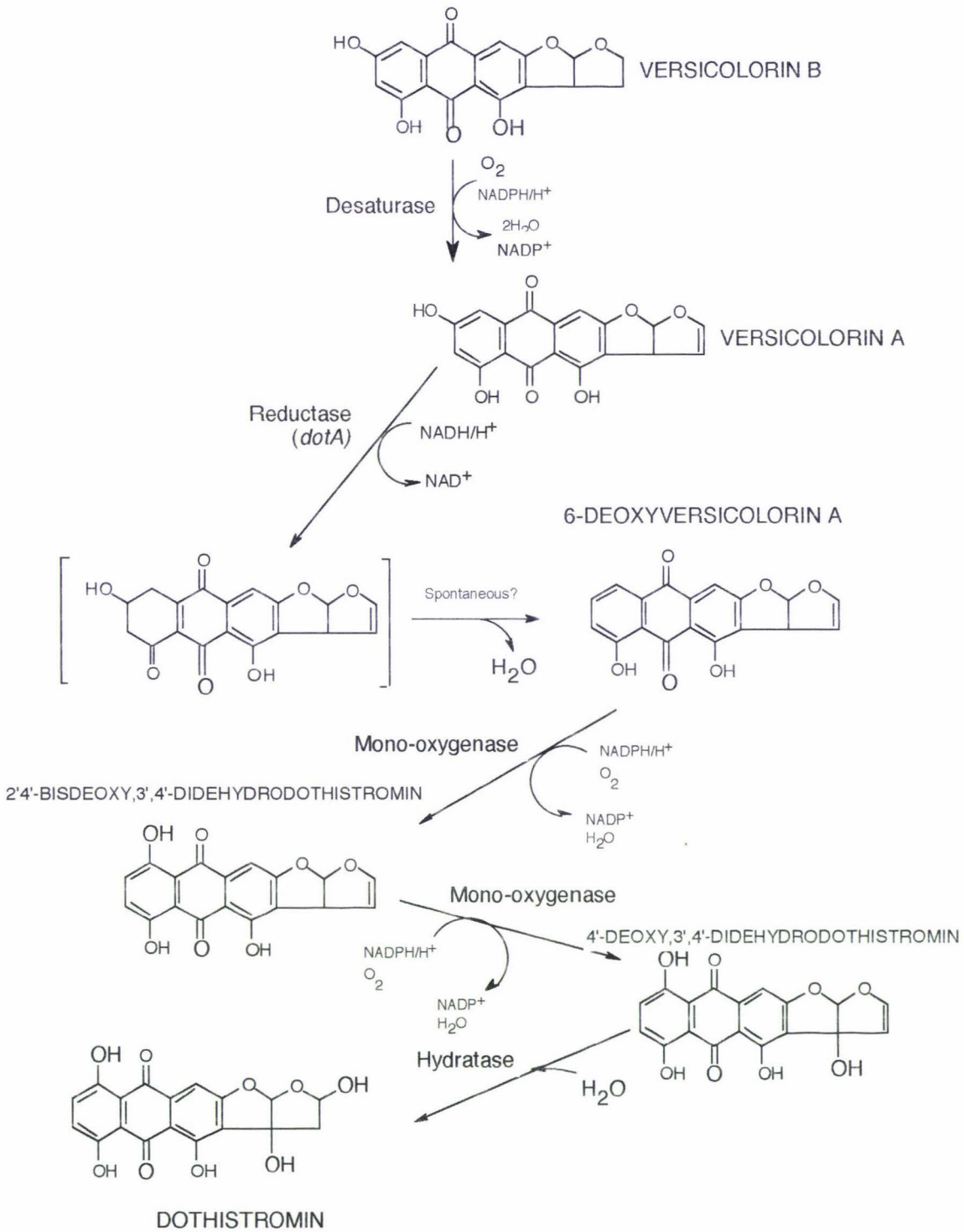
Comparison of the wild type NZE5 strain to the strain 8A1 observed to be producing excessive pigment when on solid media (table 3.4) was made by comparing the estimated means of dothistromin production. The production of dothistromin was not significantly different between the two strains at day nine when standardised to mycelial weight, however to confirm this result, more samples need to be taken and the media put onto ELISA plates undiluted so the readings are within the linear range of the standard curve. Previous studies diluted NZE5 media in order for the samples to be in the linear portion of the standard curve (Ganley 2000). It has been observed that dothistromin concentration in the medium decreases from 7 –10 days in wild type strains (Ganley 2000) and it was suggested degradation could be due to metabolic or photolytic degradation. Once

standardised to mycelial dry weight there is no statistical difference between dothistromin production at 9 days when grown in liquid culture. The higher level of dothistromin detected in the 8A1 media at 9 days could be attributed to more mycelia in the flask.

The growth rate of strain 8A1 on solid AMM + 2% glucose is significantly less than wild type (*t* test results not shown) but it can be qualitatively observed that more dark brown or black pigment accumulates on plates containing 8A1. It is possible that this pigment is not dothistromin, or it is dothistromin that has been altered so in the ELISA some is not able to be detected.

t testing of means of dothistromin production standardised to dry weight of mycelia between the strains NZE5 and 8A1 does not indicate any difference in dothstromin production, although more samples need to be taken and tested before conclusive evidence is provided (Discussed in more detail in section 3.5.4). If further work concludes that 8A1 is an overproducer of dothistromin, it could be that the integration event is in or adjacent to a gene that affects dothistromin production or disrupts a gene involved in the degradation of dothistromin. The 'tagging' of the disrupted regions within colony 8A1 with the *hph* cassette will allow the disrupted gene to be isolated using inverse PCR and *hph* specific primers.

Figure 3.13 Putative Dothistromin Biosynthetic Pathway (from Versicolorin B).



4.0 ANALYSIS OF λBMKSA: A CLONE CONTAINING PUTATIVE DOTHISTROMIN GENES.

Evidence supporting a cluster of dothistromin biosynthetic genes includes four putative dothistromin biosynthetic genes found within a 10 kb area and contained in the clone λCGV1 (Monahan 1998). One of these, *dotA*, has been confirmed to be involved in dothistromin biosynthesis (This study). The similarities between AF, ST and the putative dothistromin biosynthetic clusters, suggest that there are still more dothistromin biosynthetic genes that exist in close proximity to each other within the genome. λBMKSA is another clone thought to contain part of the dothistromin biosynthetic gene cluster. This clone will be further sequenced and analysed in this chapter. Clusters of biosynthetic genes for secondary metabolites in filamentous fungi are well documented and discussed in more detail in section 1.4.2.

4.1 INTRODUCTION

Dothistromin is a polyketide and like all polyketides including AF and ST, the first step of polyketide synthesis is catalysed by the highly conserved enzyme polyketide synthase (PKS) (section 1.4.4). As polyketide biosynthetic genes are found within biosynthetic gene clusters of many fungi, probing the genomic DNA with a conserved active site from PKS can isolate a cluster of putative polyketide biosynthesis genes. The clone λBMKSA was isolated by using the β-ketoacyl synthase (KS) domain region from the *pksA* gene of *A. parasiticus* and subcloned (Morgan 1997). One subclone was sequenced and sequence encoding conserved PKS active sites, KS and the acyl transferase (AT) domain, were revealed (Morgan 1997). Both the KS and AT domains found in the *D. pini* genome have high similarity to *A. parasiticus* PKSA (involved in AF production) suggesting the *D. pini pks* gene is involved in dothistromin biosynthesis. Further sequencing of this clone will extend the known sequence of the putative *D. pini pks* gene (pks^{dot}). Sequencing will also reveal any other putative dothistromin genes and provide further evidence to support the hypothesis that dothistromin biosynthetic genes exist within the genome as a cluster.

The putative dothistromin gene *dotD* found in the λCGV1 cluster (Monahan 1998)has high identity and similarity to the thioesterase (TE) domain sequences of *pksA* (*A. parasiticus)* and *stcA* (*A. nidulans)* (Section 1.4.3 and Table 1.1). TE domains are usually part of a single PKS polypeptide but PKSs without TE domains do exist. Examples of PKSs without TE domains are PKS1 from *C. lagenarium* and WA from *A. nidulans* (see section 1.4.4.1). The TE domains from these PKSs are thought to be contained on a separate polypeptide. Sequencing of the entire *D. pini* PKS will tell us if the gene has a TE domain or if *dotD* might be the PKS^{DOT} TE domain on a separate ORF. Further sequencing of PKS will also provide enough sequence to construct a disruption vector, that can be used to generate a pks^{dot-} mutant by gene disruption. This would confirm if PKS^{DOT} is on the dothistromin biosynthetic pathway. This study will focus on further sequencing of the λBMKSA.

4.2 SEQUENCING OF λBMKSA.

Prior to this study, subclone pR156 (a 2.4 kb fragment of the 15 kb λBMKSA clone) had been completely sequenced on both strands ((Morgan 1997), (Laarakkers 1999) and universal pUC forwards & pUC reverse primers had been used to initiate sequencing of subclone pR163 (Laarakkers, 2000). Sequencing obtained prior to this study is identified in figure 4.1 with blue arrows and summarised in figure 1.4.

4.2.1 Outline of Sequencing and contig construction.

The subclone inserted in the vector pR163 was completely sequenced (section 2.14) on both strands using universal forwards and reverse pUC/M13 primers, and primer walking with custom primers (Table 2.1 and figure 4.1). Subclone pR181 was partially sequenced on both strands by extension from the vector sequences with custom primers (Figure 4.1).

PCR was used to determine overlapping sequences between R156 & R163, R163 & R181 and R156 & λ 9 kb arm and consequently also determined the orientation of the subclones with respect to each other. The overlap between R156 and R163 was determined by amplifying genomic DNA with the primers SLpks4 and R163ovlp and sequencing the PCR product. The PCR product was approximately 300 bp larger than expected. Sequencing of the PCR product confirmed it was the overlap and revealed that the overlap contained two

EcoRI restriction endonuclease sites 300 bp apart and the 300 bp fragment had not been detected previously. The overlap between R163 and R181 was confirmed by amplifying genomic DNA with primers R181fwdsch and dmoC3, and then sequencing the product on both strands. The final fragment, between the subclone contained by R156 and the λ 9 kb arm, was amplified using primers Dpks2 & SP6 (to anneal to the lambda arm) cloned (pR209) and was sequenced on both strands. All PCRs used conditions described in section 2.13.1. A contig (Figure 4.1) was constructed with R181, R163, overlaps together with R156 (sequenced by Morgan, 1997) using the Gene Jockey II computer program (BIOSOFT, Cambridge, UK). The 7.3 kb contig sequence showing primer positions can be found in Appendix 2.

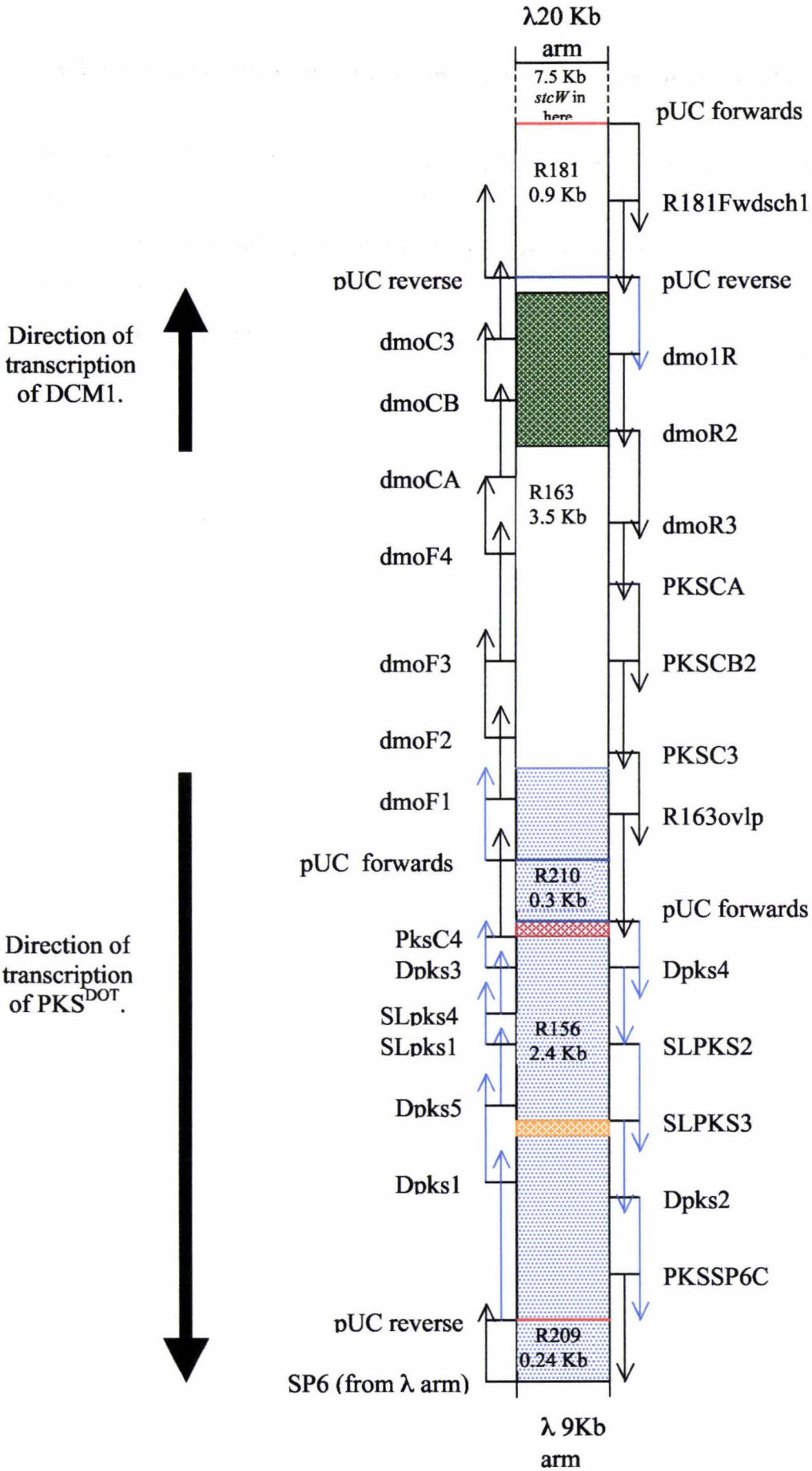
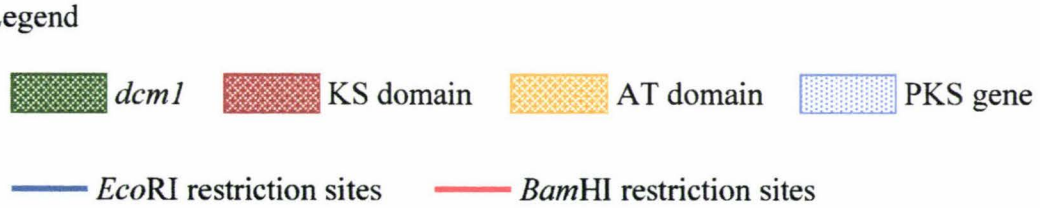

λ20 Kb
arm
7.5 Kb
stcW in
here
pUC forwards
R181
0.9 Kb
R181Fwdsch1
pUC reverse
pUC reverse
dmoC3
dmo1R
dmoCB
dmoR2
dmoCA
R163
3.5 Kb
dmoR3
dmoF4
PKSCA
dmoF3
PKSCB2
dmoF2
PKSC3
dmoF1
R163ovlp
pUC forwards
R210
0.3 Kb
pUC forwards
PksC4
Dpks3
Dpks4
SLpks4
SLpks1
R156
2.4 Kb
SLPKS2
Dpks5
SLPKS3
Dpks1
Dpks2
PKSSP6C
pUC reverse
R209
0.24 Kb
SP6 (from λ arm)
λ 9Kb
arm
Direction of
transcription
of DCM1.
Direction of
transcription
of PKS^DOT.

Figure 4.1 Double stranded sequencing strategy for the contig constructed in this study.

Figure shows direction and approximate location of sequencing primers used for the double stranded sequencing of the contig. Arrows indicate direction of the sequencing the primers are indicated by labels adjacent to arrows prime at the beginning of the arrow. Blue arrows indicate the sequencing was carried out in previous studies and black arrows indicate the sequencing carried out in this study. Specific location and sequence of primers can be found in appendix 2.



4.3 ANALYSIS OF PARTIAL λBMKSA SEQUENCE.

Sequence analysis of the contig (figure 4.1) revealed an incomplete sequence of a putative PKS gene (*pks*^{dot}(1-1426)). In addition to this, blast analysis of the sequence in the subclone R163, revealed an ORF (*dcm*1) with significant homology to known p450 cytochrome monoxygenases. Table 4.1 shows the sequence identities and similarities to genes homologous to the two ORFs in the contig.

4.3.1 Sequence Analysis of *pks*^{dot} (1-1426).

Sequencing of the subclone R163 and the overlap between R156 and R163 revealed 550 amino acids upstream to the sequence revealed in previous studies including the N terminal and the promoter region of the *pks*^{dot}. Predicted nucleotide sequence in figure 4.3 contains 5' untranslated nucleotide sequence and the coding sequence for the first 1426 amino acids of PKS^{DOT}.

4.3.1.1 Analysis of Amino Acid Sequence Predicted from Partial *pks*^{dot}.

Alignment of the partial PKS^{DOT} to homologous amino acid sequences (Figure 4.2) along with BLAST analysis supported the hypothesis that PKS^{DOT} is a PKS involved in dothistromin production. The first 1426 amino acids have 62% identity (74% similarity) to PKSA, a PKS involved in AF synthesis in *A. parasiticus*, and 63% (74%) to STCA, a PKS involved in ST synthesis in *A. nidulans* (Table 4.1).

4.3.1.2 PKS^{DOT} Contains Highly Conserved Regions.

Analysis of the amino acid sequence made available by the additional sequencing in this study did not reveal any new conserved active sites common to PKSs. Existing sequence had already revealed two conserved domains, KS and AT. These domains have been labelled on the amino acid sequence in figure 4.3.

Table 4.1 Homologous genes to putative dothistromin genes *pks*^{dot} and *dcm1*.

λBMKSA ORF	Homologous gene and organism	% identity (% similarity)	Putative function of homolog	Accession number	Reference
pks^{Dot} (partial)	*A. parasticus* *pksA*	62 (74)	Polyketide synthase (PKS), AF biosynthesis	Q12053	(Chang, Cary et al. 1995)
	A. nidulans *stcA*	63 (74)	PKS, ST biosynthesis	Q12397	(Yu and Leonard 1995)
	A. fumigatus *Alb1(pksP)*	42 (58)	PKS, AF biosynthesis	AF025541	(Tsai, Chang et al. 1998)
	A. nidulans *wA*	42 (58)	Green conidial pigment synthase.	Q03149	(Mayorga and Timberlake 1992)
dcm1	*A. parasiticus* *cypX*	59 (74)	Cytochrome p450 monooxygenase, AF.	AAF26280	(Yu, Chang et al. 2000)
	A. nidulans *stcB*	56 (68)	Cytochrome p450 monooxygenase, ST.	Q12608	(Brown, Yu et al. 1996)

Figure 4.2 Alignment of Partial PKS^{DOT} Amino Acid Sequence with PKSA from *A. parasiticus* and STCA from *A. nidulans*.

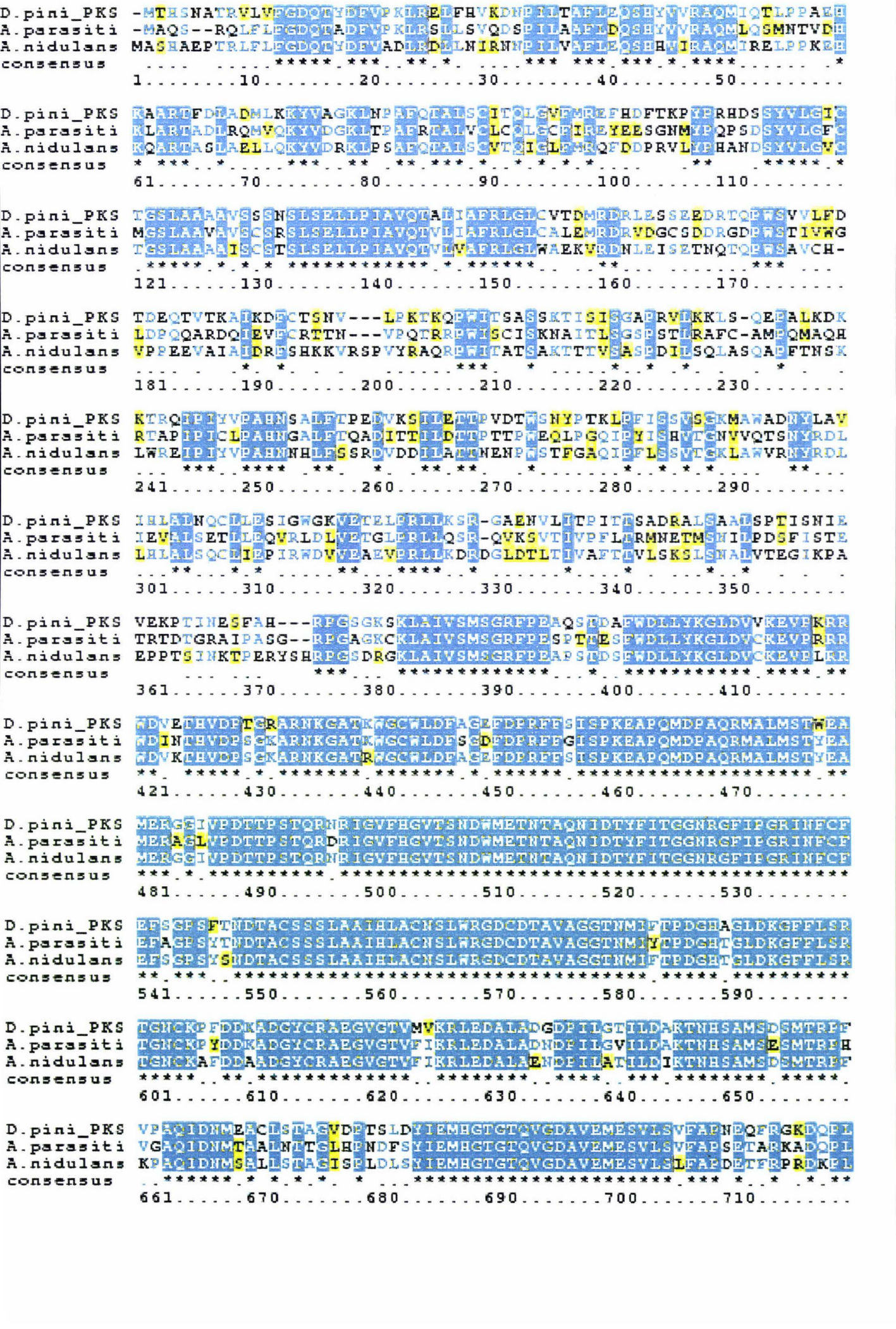
This alignment was constructed using Bionavigator (www.eBioinformatics.com).

FGD
* * *
Identical amino acids conserved between all three amino acid sequences.

FGD
Identical amino acids conserved within two of three amino acid sequences

FGD
Similar amino acids.

Accession numbers for *stcA* (*A. nidulans)* and *pksA (A. parasiticus)* are Q12397 and Q12053 respectively.


D.pini_PKS
A.parasiti
A.nidulans
consensus


D.pini_PKS
A.parasiti
A.nidulans
consensus

Figure 4.3 Partial Nucleotide and Amino Acid Sequences of PKS^{Dot}.

The portion of the existing λBMKSA contig containing sequence for the putative *pks^{Dot}*.
Numbering corresponds to the full contig sequence listed in Appendix 2.
Coloured portions of sequence correspond to consensus sequences as listed below.

Nucleotide consensus sequences

- Putative translation initiation sequence (Kozak consensus sequence)
- Putative aflR binding sites
- Putative translation start codon
- 5' fungal consensus intron splice site
- 3' fungal consensus intron splice site

Amino Acid conserved sequences

- Ketosynthase domain
- Acyltransferase domain

Nucleotides 2000 to 4610 sequenced in this study.

```
CATGGGGCCATACTTTTGGTGCAGTGAATCAACATAGTAGATGCGGTTGCCAGCGAAGACGGAGTACGTCAGGCGCAA 2078
GTCTGTCAACTTGGCATACCATGGTCCTGGAATCTTGCTCAGAGGAGAAAAGTATGCTGTCGTGATGATCTGCAACAT 2156
CTATTAGCAGCGTTTCCACCGCGAAAATTGACGTCTACTCACAGAAACAATGTTATACAGAACAAACGCAGCAGCGAC 2234
AAGTGCCAGCGAAAACGGCAATGGAGCACCAGCAGTGGCGTCCATAATCCACTTGTAGAGCTCTCCTGCCATGGTGTC 2312
GGAGTGGCAGTGAAGACCAGCAGACGTTGAAAGAGAATGACAAACGGCGTCAAGAACAGTAAGTAACTTGTCAATGCT 2390
ATCATGCAATCCGTTGATACTACACAAGCCTCGCAGAACGACTTGTCATCAACAAAGCGGTATTCCGCACGCTCTGAG 2468
TGATCCTTATGTCGGTTTGCGATACTTCGGACTTCAGGCTCGGTCAAGTGAATCCAGCCCGAAACAGCCAGCGCAAGC 2546
CCACGGCAGTACCCGATTGGATCGATTGATCGATACCGCGCAAACTGCTGCTTGCCGTCGGCCTGCACGTGTATGCCA 2624
TGCGAATGCGCTAACTCTGCCTCAGCTATCGTAATCGCCAAACCCTTCGGCCTGGCCAGCCGTCCTCACAGTGGCCCG 2702
ACTGTTGCTGGCGGACAGCGACCGTATCTCGCAGGCAAGACCCGAAGACAAATTGACTTTGATACGCAGGCCCAGTAC 2780
ACTTTCCTCGCTGTCTTCCTCGACCTGTTGACTGCCATCGATCCCAGCACCGCTCACCATACAGGCTGTCCCCTGTCT 2858
CTACACCTACACTCATTCTTTCCAGGTGTGCTTTGCGCCTCGTTGATGACACACTAATTTCGATACTCAGGCGACTTT 2936

CTTCGCCATAACCTCCCCACACAGTCAATATGACACACTCCAACGCAACGAGGGTACTGGTCTTTGGTGACCAGACCT 3014
                          M  T  H  S  N  A  T  R  V  L  V  F  G  D  Q  T  Y

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  D  F  V  P  K  L  R  E  L  F  H  V  K  D  N  P  I  L  T  A  F  L  E  Q  S  H

ATTATGTCGTGTAAGTCGTACTGATCGATCGGACGCAGACCGTTGCTAAGAATTGCCAGCCGAGCACAGATGATCCAG 3170
  Y  V  V                                                    R  A  Q  M  I  Q

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CTGAACCCTGCCTTCCAGACGGCCCTCAGCTGCATCACACAACTCGGTGTGTTCATGCGAGAGTTCCATGACTTCACC 3326
L  N  P  A  F  Q  T  A  L  S  C  I  T  Q  L  G  V  F  M  R  E  F  H  D  F  T

AAGCCATATCCACGACACGATAGCAGCTACGTGTTGGGTATCTGCACCGGCTCGCTTGCCGCTGCGGCCGTCAGCTCC 3404
K  P  Y  P  R  H  D  S  S  Y  V  L  G  I  C  T  G  S  L  A  A  A  A  V  S  S

AGCAACTCTTTATCCGAGCTTCTGCCTATTGCCGTGCAAACGGCTTTGATCGCCTTTCGCCTCGGTCTGTGCGTCACA 3482
S  N  S  L  S  E  L  L  P  I  A  V  Q  T  A  L  I  A  F  R  L  G  L  C  V  T

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D  M  R  D  R  L  E  S  S  E  E  D  R  T  Q  P  W  S  V  V  L  F  D  T  D  E

CAGACTGTTACCAAGGCCATCAAAGACTTCTGCACATCCAACGTCCTCCCGAAGACCAAGCAGCCCTGGATTACCTCC 3638
Q  T  V  T  K  A  I  K  D  F  C  T  S  N  V  L  P  K  T  K  Q  P  W  I  T  S

GCGTCATCGAAGACAATCTCCATCAGCGGAGCACCACGTGTGCTGAAGAAGTTGTCGCAAGAGCCTGCGCTCAAGGAC 3716
A  S  S  K  T  I  S  I  S  G  A  P  R  V  L  K  K  L  S  Q  E  P  A  L  K  D

AAAAAGACCAGACAGATCCCGATTTATGTCCCAGCACACAATTCTGCCCTCTTCACCCCCGAAGATGTCAAGTCTATC 3794
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GCTTGGGCAGACAACTACCTTGCAGTAATCCATCTCGCCCTCAATCAGTGCCTGCTCGAGAGCATTGGCTGGGGTAAG 3950
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GACCGTGCTCTGTCGGCTGCACTCAGCCCAACGATCTCCAATATCGAGGTCGAGAAGCCCACAATCAACGAATCTTTC 4106
D  R  A  L  S  A  A  L  S  P  T  I  S  N  I  E  V  E  K  P  T  I  N  E  S  F

GCCCATAGACCTGGTTCGGGAAAGAGCAAGCTTGCTATCGTCTCAATGTCTGGTCGCTTCCCAGAGGCACAAAGCACC 4184
A  H  R  P  G  S  G  K  S  K  L  A  I  V  S  M  S  G  R  F  P  E  A  Q  S  T

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CACGTCGACCCAACTGGGCGTGCTCGCAACAAGGGTGCGACCAAATGGGGCTGCTGGCTCGATTTCGCCGGAGAATTC 4340
H  V  D  P  T  G  R  A  R  N  K  G  A  T  K  W  G  C  W  L  D  F  A  G  E  F

GACCCTCGCTTCTTCAGCATCTCACCCAAGGAAGCACCACAAATGGACCCCGCCCAGCGTATGGCTTTGATGTCGACC 4418
D  P  R  F  F  S  I  S  P  K  E  A  P  Q  M  D  P  A  Q  R  M  A  L  M  S  T
```

```
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D  G  D  P  I  L  G  T  I  L  D  A  K  T  N  H  S  A  M  S  D  S  M  T  R  P

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F  V  P  A  Q  I  D  N  M  E  A  C  L  S  T  A  G  V  D  P  T  S  L  D  Y  I

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GTCACCAGTTTGATCAAGGTCCTTCTCATGATGCAGACCAACCACTATCCGCCGCATTGCGGTATCAAGCCTGGAAGC 5276
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GAGGGCAAGTTAAGACGGGTTTTGATCAATAACTTCAGTGCTGCAGGTGGCAATACTGCGCTTCTCATTGAGGATGCG 5432
E  G  K  L  R  R  V  L  I  N  N  F  S  A  A  G  G  N  T  A  L  L  I  E  D  A

CCTGACAGGATGCCGCTCTCAGGACAAGATCCTCGCACGACTCAGACTGTCACGATCTCGGGACATGTTGGCAAGTCT 5510
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GTCAGTGCACGAAGATGGCATCACCTCCATCGTGTTGCTGTCGCGGGTACTACCGTCGCAGATATTACCGCGAAGTTG 5666
V  S  A  R  R  W  H  H  L  H  R  V  A  V  A  G  T  T  V  A  D  I  T  A  K  L

GAGAAAGCCATTGAGAATAAGGAAGGTGTCAACAGACCTAAGGCGAAGCCTTCGGTCTTCTTCGCCTTCACAGGTCAA 5744
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GGATCTCAGTACCTCGGCATGGGCAAGCAACTCTACGACTCTTATCCAATGTTCAGATCCGAGCTTCAAGGCTACGAT 5822
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S  E  W  S  L  A  E  I  T  A  G  K  N  V  E  V  A  C  V  N  G  P  E  D  T  V

CTCTCCGGCACTGTCGAGGAAATTGGAGAGGTGCAGAAGACACTCATTGCGAAGAGCATCAAGGCTACACTCTTGAAG 6290
```

```
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L  P  F  A  F  H  S  A  Q  V  Q  P  I  L  R  G  R  S  E  E  L  A  A  G  A  T

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V  A  L  P  T  L  K  D  K  G  P  D  V  W  Q  N  L  S  N  I  F  T  T  L  Y  T

GGTGGTTTAGACATCAACTGGACTGCCTTCCACGCCCCCTTCGAGCCCGCGAAGAAGGTCCTGCAACTTCCTGATTAT 6758
G  G  L  D  I  N  W  T  A  F  H  A  P  F  E  P  A  K  K  V  L  Q  L  P  D  Y

GGCTGGGATCTCAAGGATTACTTCATCCAGTATGAAGGCGATTGGGTTCTGCATCGGCACAAGATCCACTGCAACTGT 6836
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CCTGGTGGGGCTCAGAAGGCCGTTCAGGAAGCACCTGCGGCGAAGACAGAGACGAAGAAGATGTCGAAGCTGGATCCT 6992
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CCCCTCTGCACGCCATCATTCTATGCGGATATTGCGCTTCAGGTTGGCAAATACGCCATGGACCGCATCCGTGCTGGA 7226
P  L  C  T  P  S  F  Y  A  D  I  A  L  Q  V  G  K  Y  A  M  D  R  I  R  A  G

CATCCCGGTGCCGGTGCTATTGATGGAAGGGTCGATGTTACGGATCGAG 7275
H  P  G  A  G  A  I  D  G  R  V  D  V  T  D  R
```

4.3.2 Construction of *pks*^{dot} disruption vector.

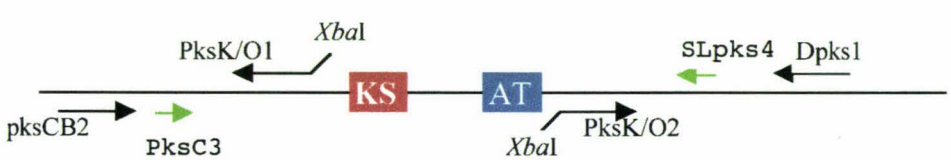
Disruption of *pks*^{dot} will show whether or not the gene is involved in dothistromin biosynthesis. Targeted disruption will be attempted according to a scheme based on the disruption of the *dotA* gene (Steps 1-5, figure 3.1). This involves transformation of wild type protoplasts with a specifically constructed disruption vector and disruption via homologous recombination. Work towards the construction of a *pks*^{dot} disruption vector is described below.

Step 1 Amplification of 5' and 3' Flanking Regions.

Specific primers were used to amplify regions flanking the conserved domains KS and AT. It is thought that replacement of these two domains with the 2.3 Kb *hph* cassette will make the *D. pini* PKS non-functional.

5' and 3' flanking regions were amplified as described in section 2.13.1 except that only 0.1 mM dNTPs were used per reaction and 2-5 ng of plasmid DNA was used as template. Primers pksK/O1and pksCB2 were used to amplify genomic DNA to give a 1.4 Kb 5' flank. Primers pksK/O2 and Dpks1 were used to amplify plasmid R156 DNA and give a 1.5 Kb 3' flank (Figure 4.4).

Figure 4.4 Amplification of genomic regions flanking the KS and AT domains.



4.3.1.3 Existing sequence of *pks*^{dot} has only one intron

The predicted amino acid sequence of the *pks*^{dot} gene was aligned with homologous proteins. The alignment and the positions of conserved intron splice sites (5' [GT(A/G/T)NGTY] and 3' [YAG] within the nucleotide sequence, indicates that within the first 1426 amino acids there is only one intron. The conserved 5' splice site (GTAAGTC) and the 3' splice site (CAG) are shown on figure 4.3.

4.3.1.4 *pks*^{dot} Promoter Region Contains Fungal Consensus Sequences.

The start site of *pks*^{dot} was predicted by comparing translation start sites in the multiple alignment with homologs PKSA and STCA, and through the presence of a conserved nucleotide sequence associated with fungal translation start sites. Fungal genes often have a conserved translation initiation sequence, the Kozak sequence CNNNCA(A/C)NATGGC (Bruchez and Eberle 1993).The predicted start site of *pks*^{dot} has a Kozak sequence beginning at –8 and incorporating the ATG start codon (CAGTCAATATGAC).

Analysis of the 5' untranslated region of the putative *pks* gene did not reveal a TATA box for transcription initiation nor does it contain a CCAAT consensus seen in other eukaryotes.

Potential binding sites for the *A. nidulans* ST regulatory protein AflR ($TCGN_5CGA$) (Fernandes *et al,* 1998) are evident at 486 and 545 bp upstream of the predicted start codon although the putative dothistromin biosynthetic regulatory motif found in other putative dothistromin genes ($TCGN_{11}CGA$) (Astin 2000) is not present.

Step 2 Combining the 5' and 3' Flanking Regions (Figure 3.2).

This step is equivalent to the step described in figure 3.2 for *dotA* except using the following primers (primers are listed with their *dotA* counterparts). *dkr1A = pksK/O 1, dkr1B = pksCB2, dkr1C = Dpks1, dkr1D = pksK/O 2.*

A Qiagen PCR purification kit was used to purify the PCR products resulting from amplification of the genomic DNA. These purified products were used in approximately equal amounts (5 ng of each flank per reaction) as the template for a PCR reaction using primers pksCB2 and Dpks1. Primers pksK/O1 and pksK/O2 were designed with *Xba*I tails. These tails allowed the 5' and 3' flanking regions to be amplified to give one 2.9 kb PCR product when amplified with primers pksCB2 and Dpks1 (Figure 3.1 for schematic illustration of overlapping *Xba*I tails). PCR conditions used were the same as those described in section 2.13.1. with the following changes; 0.2 μM of each primer was used and in the thermal cycler programme the denaturing step was 30 seconds and the annealing step took place at 49°C. Changes were made after optimisation of the PCR conditions to decrease multiple bands and increase the specificity of the primers to produce the desired 2.9 kb product.

Step 3 Ligation of 2.9 kb Hybrid into pGEM-T Vector.

After gel extraction (section 2.6.1) of the 2.9 Kb product (the 1.4 Kb and 1.5 Kb flanks amplified together), two products (2.9 kb and 1.5 kb) were still seen. To isolate a plasmid containing only the 2.9 kb fragment, this mixture of bands was ligated into pGEM-t (Promega) following the protocol recommended in kit guidelines. An insert to vector ratio of 6:1 was used to increase the probability a 2.9 kb product would ligate into the vector.

The ligation reaction was transformed into XL-1 *E. coli* (method 2.2.3). Transformed *E. coli* were screened for recombinant plasmids using a PCR screen. with primers PKSC3 and SLpks4 (positions shown on figure 4.4). The primers were contained within the flanking

regions and would only produce a PCR product if the desired 2.9 kb PCR product was ligated into the vector. PCR conditions are described in section 2.13.3 One colony produced the desired product and the plasmid was extracted using a Qiagen plasmid preparation minikit and named pR211.

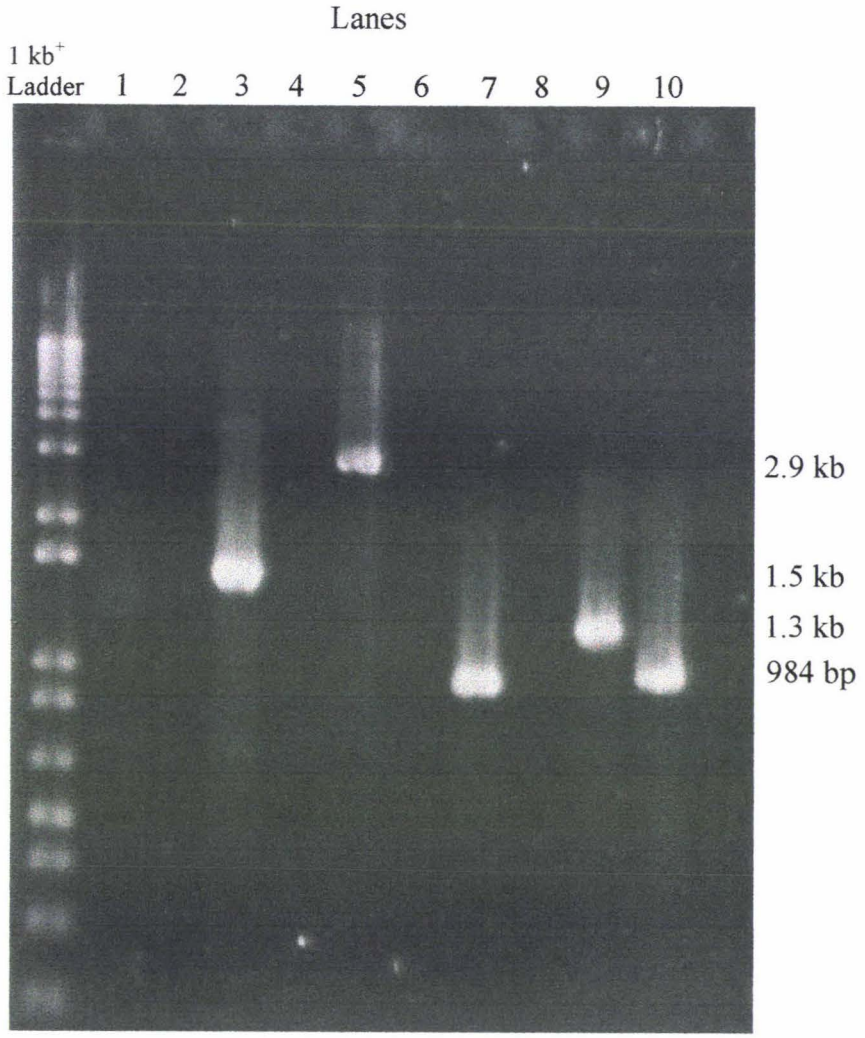
PCR was used to determine the presence and orientation of the flanking regions within the pGEM-T vector. Primers annealing in each orientation (pksK/O1 and pksK/O2) were each used in combination with the primer pGEM T7. PCR was performed according to section 2.13.1. All amplifications resulted as expected and confirmed the vector contained the desired insert in orientation two (See Figure 4.5). Since the combination of pksK/O2 & T7 gave product whilst pksK/O1 & T7 did not. Moreover, amplification with SP6 and T7 confirmed a 2.9 kb insert is contained within the vector.

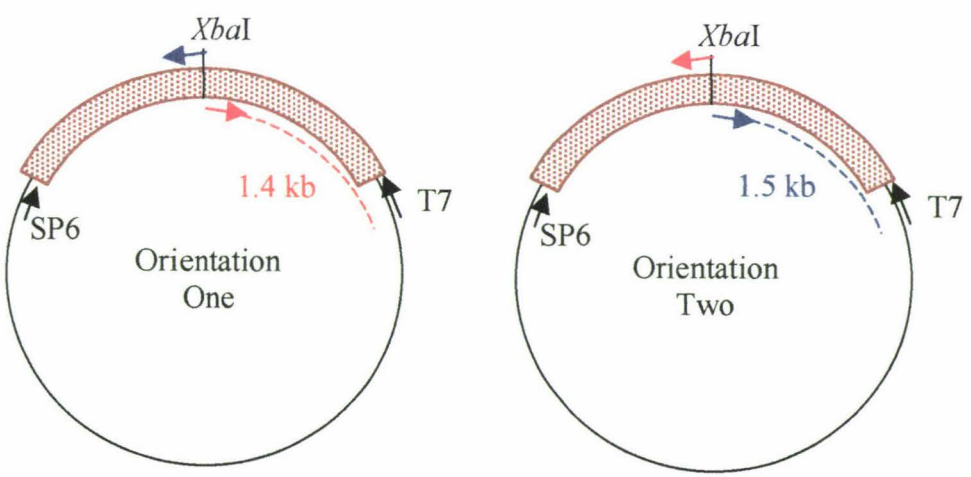
Multiple attempts at digesting plasmid pR211with *Xba*I in order to insert the *hph* cassette were unsuccessful. Appropriate positive controls showed the three different stocks of *Xba*I tried were digesting successfully But a number of digestions of pR211 using the *Xba*I from these different sources were all unsuccessful suggesting the lack of an *Xba*I restriction site within the plasmid. PCR analysis (figure 4.5) provided evidence that supported the integrity of the sequence of the insert and showed the primer sequences pksK/O1 and pksK/O2 (primers with the *XbaI* tails) were contained in the insert, suggesting the original amplification produced the expected amplicons. *Xba*I digestion is inhibited by the presence of N^6-methyladenine or 5'methylcytosine. Methylation of the DNA at the *Xba*I restriction site could be preventing digestion. The insert was sequenced with the primer SLpks4. The sequence showed signals that were very low and the presence of the *Xba*I site was unable to be confirmed. The experiment was stopped at this point in order to focus on the analysis of *dotA*$^-$ mutants.

Figure 4.5 PCR amplifications to check insert orientation within pR211 and presence of primer binding sites.

Orientation was determined using two sets of primers. One primer in each set, T7, anneals to the vector on one side of the insertion site. The other of the pair anneals to the *D. pini* DNA. A product from one of these primer sets will determine the orientation of the insert within the vector. PCR amplification with T7 and SP6 (primers anneal the vector on either side of the insert) will confirm that the insert is the correct size. Amplification with the primers pksC3 and SLpks4 will confirm annealing sites for these primers are contained within the sequence of the insert. As a positive control, the original 2.9 Kb insert was amplified with the same primers.

Lane	PCR reaction	Expected amplicon	Expected result achieved?
1	T7, pksK/O 1 and R211	1.4 Kb (if this orientation)	Nothing
2	T7, pksK/O 1 and H_2O		Nothing
3	T7, pksK/O 2 and R211	1.5 Kb (if this orientation)	Yes
4	T7, pksK/O 1 and H_2O		Nothing
5	T7, SP6 and R211	2.9 Kb	Yes
6	T7, SP6 and H_2O		Nothing
7	PKSC3, SLpks4 and R211	984 bp	Yes
8	PKSC3, SLpks4 and H_2O		Nothing
9	T7, SP6 and +ve pGEM control.	1.3 Kb	Yes
10	PKSC3, SLpks4 and original 2.9 Kb fragment.	984 bp	Yes





PksK/O1 PksK/O2 2.9 kb insert pGEM-T vector

4.3.3 Sequence analysis of *dcm1*

Sequencing of the λBMKSA clone revealed another open reading frame (ORF) thought to be involved in dothistromin biosynthesis. This ORF was identified by BLAST analysis and found to be homologous to genes in the family of cytochrome p450 monooxygenases. The ORF displays the highest amino acid sequence similarity to cytochrome p450 monooxygenases involved in AF biosynthesis (*A. parasiticus cypX*, identity 59% (similarity 74%) and ST biosynthesis (*A. nidulans stcB* 56% (68%) (figure 4.6). The high sequence similarity suggests the involvement of the gene product in dothistromin biosynthesis. For this reason it has been tentatively named *dcm1* for dothistromin cytochrome p450 monooxygenase. The nucleotide and amino acid sequence of *dcm*1 can be seen in figure 4.7.

4.3.3.1 Conserved amino acid sequences within *dcm1*.

The high amino acid sequence similarity extends further than just *cypX* and *stcB* to other members of the cytochrome p450 monooxygenase family. Three highly conserved amino acid motifs are evident in all cytochrome p450 enzymes. The major motif is a heme binding ligand (F--G---C-G) and is evident in *dcm1* (FGQGSRQCLG). The other two motifs are the K-helix for H bonding with the neighbouring sequence (E--R) present in *dcm1* as ESMR and the putative oxygen-binding pocket called the I-helix present in all p450 cytochrome mono-oxygenases as (A---T) present in *dcm1* as AATLT.

4.3.3.2 Conserved nucleotide sequences within *dcm*1.

The start site was determined by the extent of the longest ORF and by comparison of amino acid sequences to *cypX* and by BLAST analysis and lacks a completely conserved fungal predicted translation initiation sequence (Kozak sequence). There is a perfect Kozak sequence at –160 but translation initiated by this start codon would be prematurely terminated, due to two termination codons just prior to the translation start site of *dcm*1. Careful examination of the sequences, has indicated this is not a sequencing error. The 7 bp immediately upstream of the predicted start site ATG do contain some conserved Kozak bases but 4 of these 8 conserved bases are 'N' and 3 more are the start codon.

Figure 4.6 Multiple Alignment of *dcm1* and homologs *cypX* from *A. parasiticus* and *stcB* from *A. nidulans*.

A. parasiticus cypX gene contains 2 introns, one at amino acid 37 (67 base pairs long) and the other at amino acid 423 (55 bp long).

D. pini dcm1 gene contains 1 intron at amino acid 363 (55 bp long).

A. nidulans stcB gene contains 3 introns, none of which are conserved with either *cypX* or *dcm1*.

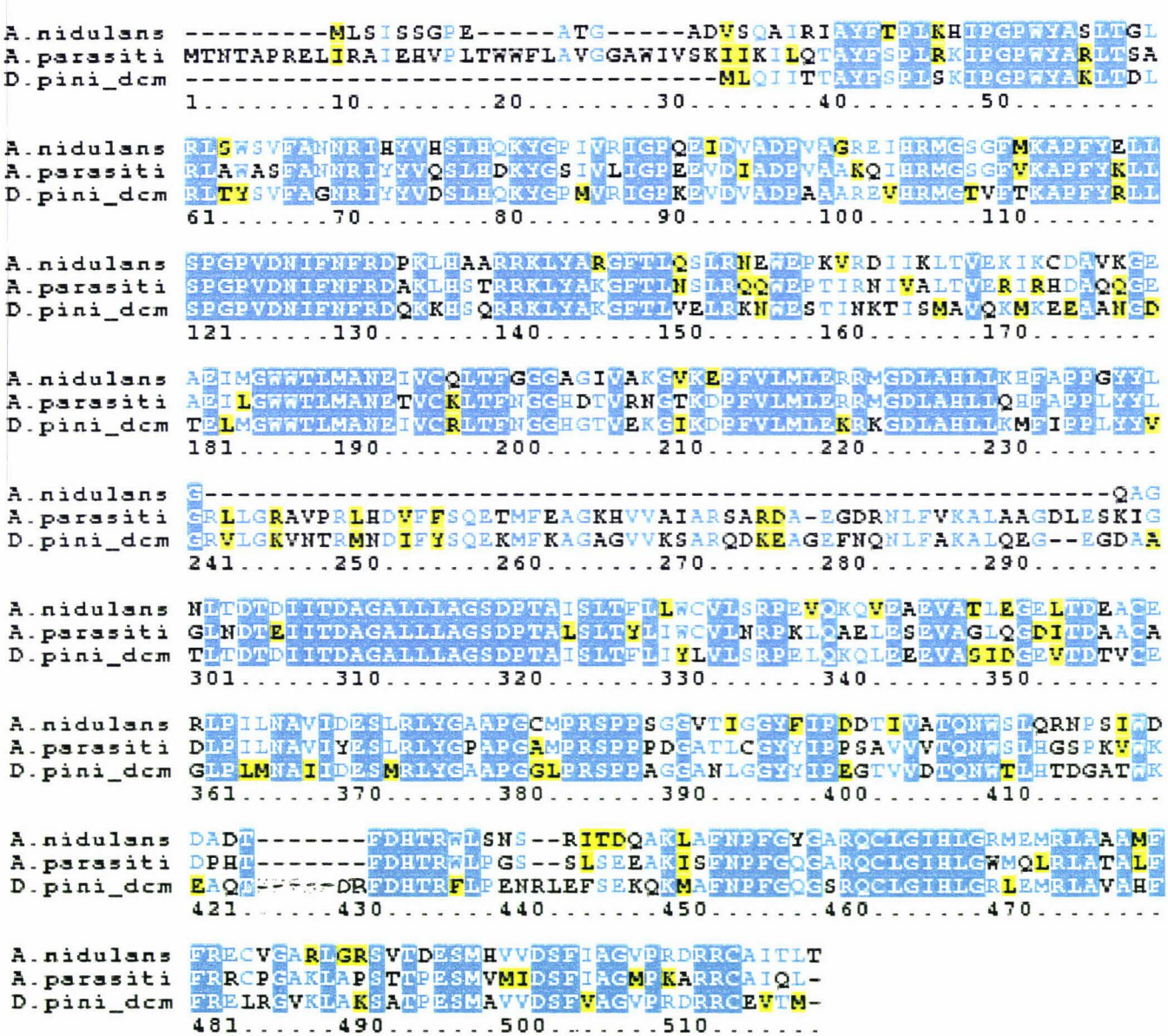

A.nidulans
A.parasiti
D.pini_dcm

Figure 4.7 Nucleotide and amino acid sequences of the putative dothistromin cytochrome p450 monooxygenase gene.

Numbers alongside correspond to the position of the nucleotide within the contig listed in appendix 2.

Consensus sequences are coloured as listed below.

Kozak sequence resulting in short ORF.
Putative aflR binding sites.
Translation start codon
5' intron splice site
3' intron splice site
Translation termination codon
CCAAT transcription activation site

Conserved amino acid sequences

Heme binding motif.
K helix for hydrogen bonding.
I helix oxygen-binding pocket.

```
AGCAGTTTGCGCGGTATCGATCAATCGATCCAATCGGGTACTGCCGTGGGCTTGCGCTGGCTGTTTCGGGCTGGATTC
ACTTGACCGAGCCTGAAGTCCGAAGTATCGCAAACCGACATAAGGATCACTCAGAGCGTGCGGAATACCGCTTTGTTG
ATGACAAGTCGTTCTGCGAGGCTTGTGTAGTATCAACGGATTGCATGATAGCATTGACAAGTTACTTACTGTTCTTGA
CGCCGTTTGTCATTCTCTTTCAACGTCTGCTGGTCTTCACTGCCACTCCGACACCATGGCAGGAGAGCTCTACAAGTG
GATTATGGACGCCACTGCTGGTGCTCCATTGCCGTTTTCGCTGGCACTTGTCGCTGCTGCGTTTGTTCTGTATAACAT

TGTTTCTGTGAGTAGACGTCAATTTTCGCGGTGGAAACGCTGCTAATAGATGTTGCAGATCATCACGACAGCATACTT 2127
                                                    M  L  Q  I  I  T  T  A  Y  F

TTCTCCTCTGAGCAAGATTCCAGGACCATGGTATGCCAAGTTGACAGACTTGCGCCTGACGTACTCCGTCTTCGCTGG 2049
 S  P  L  S  K  I  P  G  P  W  Y  A  K  L  T  D  L  R  L  T  Y  S  V  F  A  G

CAACCGCATCTACTATGTTGATTCACTGCACCAAAAGTATGGCCCCATGGTGAGGATCGGTCCCAAGGAGGTGGATGT 1971
 N  R  I  Y  Y  V  D  S  L  H  Q  K  Y  G  P  M  V  R  I  G  P  K  E  V  D  V

TGCAGATCCAGCGGCGGCACGGGAGGTGCACCGCATGGGTACAGTGTTCACAAAAGCTCCGTTCTATCGTCTCCTTTC 1893
 A  D  P  A  A  A  R  E  V  H  R  M  G  T  V  F  T  K  A  P  F  Y  R  L  L  S

TCCAGGCCCGGTTGACAACATCTTCAACTTTCGCGACCAGAAGAAGCACAGCCAGCGCCGCAAGCTGTATGCCAAGGG 1815
 P  G  P  V  D  N  I  F  N  F  R  D  Q  K  K  H  S  Q  R  R  K  L  Y  A  K  G

CTTTACTCTTGTCGAGCTGAGAAAGAATTGGGAAAGCACGATCAACAAGACCATCAGCATGGCCGTGCAGAAGATGAA 1737
 F  T  L  V  E  L  R  K  N  W  E  S  T  I  N  K  T  I  S  M  A  V  Q  K  M  K

GGAAGAGGCTGCCAATGGCGACACCGAGCTCATGGGCTGGTGGACGCTGATGGCCAACGAGATTGTCTGCCGCCTGAC 1659
 E  E  A  A  N  G  D  T  E  L  M  G  W  W  T  L  M  A  N  E  I  V  C  R  L  T

GTTCAATGGCGGACACGGAACTGTCGAGAAGGGTATCAAAGATCCATTCGTGCTCATGCTTGAGAAGCGTAAAGGCGA 1581
 F  N  G  G  H  G  T  V  E  K  G  I  K  D  P  F  V  L  M  L  E  K  R  K  G  D

CCTCGCACACCTCCTCAAGATGTTCATTCCCCCTCTTTACTACGTCGGTCGGGTACTCGGAAAGGTCAACACGCGCAT 1503
 L  A  H  L  L  K  M  F  I  P  P  L  Y  Y  V  G  R  V  L  G  K  V  N  T  R  M

GAACGACATTTTCTACTCGCAGGAGAAAATGTTCAAGGCCGGTGCTGGCGTGGTGAAGAGCGCTCGTCAGGACAAGGA 1425
 N  D  I  F  Y  S  Q  E  K  M  F  K  A  G  A  G  V  V  K  S  A  R  Q  D  K  E

GGCTGGCGAGTTCAACCAGAACTTGTTCGCAAAGGCGTTGCAGGAGGGCGAGGGCGATGCTGCGACGTTGACTGATAC 1347
 A  G  E  F  N  Q  N  L  F  A  K  A  L  Q  E  G  E  G  D  A  A  T  L  T  D  T

GGACATCATCACTGACGCCGGTGCTCTGTTGCTCGCCGGCTCCGACCCGACTGCCATTTCGCTGACGTTCCTGATCTA 1269
 D  I  I  T  D  A  G  A  L  L  L  A  G  S  D  P  T  A  I  S  L  T  F  L  I  Y

CCTCGTTCTCAGCCGACCTGAGCTCCAGAAGCAGCTGGAGGAGGAAGTCGCGTCGATCGATGGAGAAGTGACGGATAC 1191
 L  V  L  S  R  P  E  L  Q  K  Q  L  E  E  E  V  A  S  I  D  G  E  V  T  D  T

CGTATGTGAGGGACTGCCGCTGATGAACGCCATCATTGACGAAAGCATGCGACTGTACGGTGCAGCTCCCGGAGGACT 1113
 V  C  E  G  L  P  L  M  N  A  I  I  D  E  S  M  R  L  Y  G  A  A  P  G  G  L

TCCACGCAGCCCGCCAGCTGGTGGTGCCAACCTTGGTGGATACTACATCCCCGAAGGCACTGTCGTCGACACGCAGAA 1035
 P  R  S  P  P  A  G  G  A  N  L  G  G  Y  Y  I  P  E  G  T  V  V  D  T  Q  N

TTGGACTTTGCACACGGATGGTGCCACGTGGAAGGAGGCACAGACGTGAGTTGTGTATGTGTGGTGGATGCTCGGGGG 957
 W  T  L  H  T  D  G  A  T  W  K  E  A  Q  T ---------INTRON------------------

AGATGCTAATGTTGCGACAGATTCGACCACACGCGCTTCCTGCCCGAGAACAGGCTCGAATTCAGTGAGAAGCAGAAG 879
----------------------F  D  H  T  R  F  L  P  E  N  R  L  E  F  S  E  K  Q  K

ATGGCGTTCAACCCGTTCGGCCAAGGTTCCAGGCAGTGTTTGGGCATTCATCTGGGCAGGTTGGAAATGCGTCTTGCC 801
M  A  F  N  P  F  G  Q  G  S  R  Q  C  L  G  I  H  L  G  R  L  E  M  R  L  A

GTTGCTCACTTCTTCCGAGAGCTGCGCGGAGTCAAGCTGGCCAAGTCGGCGACGCCTGAGAGCATGGCGGTGGTGGAC 723
V  A  H  F  F  R  E  L  R  G  V  K  L  A  K  S  A  T  P  E  S  M  A  V  V  D

AGTTTCGTTGCCGGTGTACCGAGAGACAGGCGGTGCGAAGTGACGATGAAGGCATGAGCATGAAGGAATGGATGTTGT 645
S  F  V  A  G  V  P  R  D  R  R  C  E  V  T  M  K  A  ·

TGTGCATTGGGTGAAGTAAAGGTCGAGGCCGTGTCGTGTGTAAACGGGGAAAACGAAACACACATTGTTGCAGAGTCA 567
ATCAAGTTTTAGGGGTATCTGCACNCGATATTGATGGACTGTTTTACATCATCTTTCTCACCCACTTCCACTGCCACT 489
TCGTCTGCAGTCTTCTTCACCCGTGGTCACCT
```

GCTAATAGATGTT (underlined bases indicate those that are conserved). Apart from the start codon, only 1 base is conserved according to the Kozak sequence.

The presence of only one set of conserved intron splice sites and alignment with the homologs *cypX* and *stcB* indicate only one intron is present in the *dcm1* nucleotide sequence. A conserved fungal 5' intron splice site is present beginning at nucleotide 1080 and a 3' conserved fungal intron splice site is present beginning at nucleotide 1130. The positions of introns within *dcm1 & stcB* and *cypX & stcB* are not conserved but the intron in *dcm1* is at the same position and is the same length as the second intron in *cypX*.. The *A. parasiticus* gene *cypX* contains two introns, 67 and 55 bp and the *A. nidulans* gene *stcB* has three introns 41,146 and 71 bp long. A termination codon is present at nucleotide 1401.

Sequences matching the consensus binding sites for the *A. nidulans* regulatory protein $AflR^{ST}$ ($TCGN_5CGA$) are evident at –256 and –324. As in pks^{Dot}, putative dothistromin biosynthetic regulatory motif found in other putative dothistromin genes ($TCGN_{11}CGA$) is not present. Homolog *cypX* has the $TCGN_5CGA$ motif at –461 while the *A. nidulans stcB* does not. The TATA box (TATAAA) often found in eukaryotic promoters is not present in *dcm1* while a CCAAT consensus for transcriptional activation is present.

4.4 DISCUSSION

4.4.1 λBMKSA contains the N-terminal region of putative dothistromin PKS.

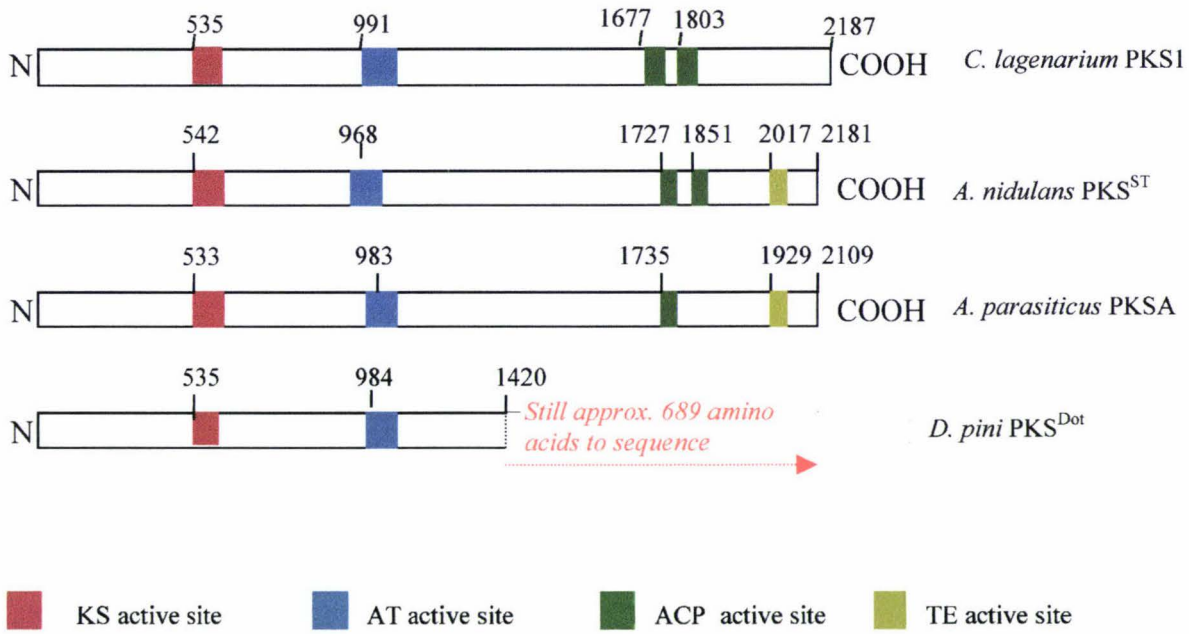
550 amino acids including the N-terminal domain of the putative PKS^{Dot} have been revealed by the continued sequencing of the λBMKSA clone in this study. The C-terminal coding region for approximately 690 more amino acids is expected to be found in another clone as the end of λBMKSA truncates the gene.

PKS^{Dot} has not been fully sequenced, but the high amino acid similarity thus far to PKSA indicates that PKS^{Dot} is a good candidate for a dothistromin polyketide synthase gene. The high sequence similarity to PKSA and PKS^{ST}, and the highly conserved nature of fungal

PKSs, provides insight into the possible structure of PKS^{Dot} suggesting that there are still approximately 700 unsequenced amino acids in the polypeptide. Although no new conserved active sites have been found, they were not expected in the N-terminal region. The two active sites not yet found by sequence analysis of PKS^{Dot} are expected (by comparison to homologous proteins, Fig 4.8) to be in the C-terminal region.

While the missing TE domain could be contained on a separate ORF (possibly the *dotD* gene found by Monahan, 1998 in previous studies) homologies suggest that at least one acyl carrier protein active site (ACP) will be found within the pks^{Dot} reading frame when it is extended and fully known. In order to isolate the clone containing the remaining pks^{Dot} nucleotide sequence, a genomic library could be hybridised with a probe based on the AT active site from the *D. pini* PKS^{DOT} to find another clone containing the AT domain and possibly the remainder of the gene.

Figure 4.8 Comparison of PKS proteins from *A. nidulans*, *A. parasiticus* and *C. lagenarium* to incomplete *D. pini* PKS.



4.4.2 Construction of a *pks*^{Dot} disruption vector.

The construction of the *pks*^{dot} disruption vector was stopped due to difficulties in digesting the vector containing the two flanking regions with *Xba*I. Sequencing will conclusively show if the restriction site is present in the insert. Due to a shift in focus to analyse the *dotA*$^{-}$ mutants, completion of the *pks*^{Dot} disruption vector was not possible in this study.

To get a clean sequence of the vector, the mixture used for sequencing (pGEM-T vector containing the disruption flanking regions) will be transformed into *E. coli* and reisolated to produce higher sequencing signals. Successful sequencing of the disruption vector will indicate whether the *Xba*I site is present. The vector can be corrected or a new vector constructed for the *hph* cassette to be inserted, making the *pks*^{dot} disruption vector. Methylation of *Xba*I restriction prevents digestion. To prevent methylation, the vector can be used to transform a methylation^{-} host *E. coli.* Transformation of NZE5 wild type protoplasts with the *pks*^{dot} disruption vector will disrupt the *pks*^{dot} gene and mutants analysed in the same way as the *dotA*$^{-}$ mutants. Disruption of putative dothistromin (*dot)* genes and analysis will enable the dothistromin biosynthetic pathway to be elucidated.

The 984 bp PCR product in lane 7 of figure 4.5 can not be amplified from a hybrid product of two 3' or two 5' flanks. Amplification with the primer pksC3 and two 5' flanks would produce a PCR product 1480 bp. Amplification with primer SLpks4 and two 3' flanks would produce a PCR product 440 bp.

4.4.3 Sequencing of λBMKSA revealed a putative dothistromin cytochrome p450 monooxygenase gene.

The putative dothistromin biosynthetic gene *dcm1* had high amino acid sequence similarity to cytochrome p450 monooxygenases *stcB (A. nidulans)* and *cypX (A. parasiticus)*. *stcB* and *cypX* are thought to be involved in ST and AF biosynthesis respectively, due to their genomic location within ST and AF biosynthetic gene clusters. Disruptants of *cypX* or *stcB* have not given conclusive evidence of their function, but like *dcm1*, they both contain the three highly conserved motifs characteristic of cytochrome p450 enzymes (see section 4.3.3.1). Mono-oxygenases are required at a number of steps after versicolorin B on the proposed dothistromin biosynthetic pathway (Figure 3.13) and a p450 cytochrome mono-oxygenase *(avnA)* catalyses the conversion of averantin to 5' hydroxy-averantin in the AF biosynthetic pathway (figure 1.2). The *D. pini* gene *dcm1* is homologous to *cypX* in the AF pathway, and is less similar to *avnA*. This suggests *cypX* and *dcm1* catalyse similar reactions. After versicolorin A, the dothistromin pathway diverges from the AF pathway. The presence of a homolog to *cypX* and *stcB* in *D. pini* indicates these genes could function in the first half of the AF and ST pathways respectively and deduction of the function of *dcm1* gene function will lead to further understanding to the functions of *cypX* and *stcB*.

The presence of an intron identical in length and position in *dcm1* and *cypX* suggests these homologs are derived from the same ancestral gene.

4.4.4 Further sequencing of λBMKSA provides supporting evidence for the presence of a dothistromin biosynthetic cluster.

The sequencing of λBMKSA has so far revealed three ORFs, all of which are putative dothistromin biosynthetic genes based on amino acid similarity to ST and AF gene products. The partial sequence of a putative PKS, pks^{dot} ((Morgan 1997);(Laarakkers 1999) and this study), a putative dothistromin cytochrome p450 monooxygenase, *dcm1* (this

study) and a partial ORF homologous to the *A. nidulans* monooxygenase *stcW* (Laarakkers 1999) have been identified. The linkage of these three putative dothistromin biosynthetic genes, indicates that the *D. pini* genes involved in dothistromin biosynthesis could be arranged within the genome as a cluster and part of this cluster is contained on the λBMKSA clone. Further sequencing of the clone will hopefully uncover further ORFs that could be involved in dothistromin biosynthesis. Verification that the λBMKSA clone contains part of the dothistromin biosynthetic cluster could be confirmed by disrupting the putative dothistromin genes and examining the mutants for dothistromin production.

With more nucleotide sequences becoming available, finding patterns important in initiation and activation of translation of dothistromin genes is possible. Motifs suggested are summarised below in table 4.2.

Table 4.2 Motifs present in the 5' regions of putative dothistromin biosynthetic genes.

	Promoter region elements			
Gene	TATA box	CAAT box	AFLR binding site	Kozak sequence
consensus	TATAAA	CCAAT		CNNNCA(A/C)NATGGC
dotA	Not present	Not present	$TCGN_{11}CGA$	CTCCCATAATGTC
dotB	Not present	CCAAT	$TCGN_{11}CGA$	Not present
dotC	Not present	CCAAT	$TCGN_{11}CGA$	CATGCGATATGGC
dotD	Not present	Not present	$TCGN_{11}CGA$	CACGCAAAATGTC CCCTCAACATGGA
pks^{dot}	Not present	Not present	$TCGN_5CGA$ (present twice)	CAGTCAATATGAC
dcm1	Not present	CCAAT	$TCGN_5CGA$ (present twice)	GCTAATAGATGTT

TATA boxes assist in guiding of RNA Polymerase II to the gene promoter (Struhl 1995). It has been shown that TATA boxes do not have a major role in determining transcription efficiency in filamentous fungi (Punt and van den Hondel 1992) therefore it is not

surprising that not all fungal genes (including most of the dothistromin biosynthetic genes to date) have this promoter element (Jacobs and Stahl 1995).

CCAAT boxes bind transcription activators in higher eukaryotes, and CCAAT binding factors have been observed in fungi (Bonnefoy, Copsey *et al.* 1995). Activator activity is not always essential for transcription initiation so its absence from some dothistromin biosynthetic genes is not surprising.

Both the AF and ST biosynthetic genes are transcriptionally co-regulated by homologous C_6 zinc cluster transcription factors, ApAflR in *A. parasiticus* (Chang, Ehrlich *et al.* 1995) and AnAflR in *A. nidulans* (Yu, Butchko *et al.* 1996). Both AflR homologs activate transcription of genes they regulate and bind to the motif $TCGN_5CGA$ [Fernandes, 1998 (Ehrlich, Montalbano *et al.* 1999) as well as other weaker sites. In ST and AF biosynthesis the AflR transcription factors don't seem to bind to earlier genes such as *pksA*. The promoter of pks^{dot} contains two perfect AflR binding sites, as does *dcm1*. Other putative biosynthetic gene promoters contain the motif $TCGN_{11}CGA$. It is thought pks^{dot} acts early in the biosynthetic pathway and it is not known where *dcm1* acts. It is interesting that adjacent genes pks^{dot} and *dcm1* promoters both contain two $TCGN_5CGA$ motifs separated by 59 base pairs. It could be they are similarly regulated and possibly both at early stages of the pathway.

5.0 CONCLUSION AND FUTURE WORK.

The main aims of this project were to elucidate *dotA* function and to further sequence λBMKSA, a clone containing putative dothistromin biosynthetic genes including sequence coding for the putative dothistromin PKS

.

No clones isolated from *D. pini* so far are large enough to contain the entire putative dothistromin biosynthetic cluster. The dothistromin cluster is expected to be approximately 60 kb like the AF and ST clusters. One portion of the dothistromin cluster is contained within the λCGV1 clone. This clone was fully sequenced and 4 of the 5 ORFs found are putative dothistromin biosynthetic or transporter genes. One of these (*dotA)* has been shown to be involved in dothistromin biosynthesis. Wild type *D. pini* protoplasts were transformed with a vector designed to disrupt the *dotA* gene. Southern blotting confirmed that colonies 32, 34Y1 and 34C1 had the *hph* cassette integrated by homologous recombination and disrupted the *dotA* locus. ELISA indicated that the *dotA*$^-$ mutants produced 10 – 43 fold less dothistromin than wild type *D. pini*, evidence that *dotA* is on the dothistromin biosynthetic pathway an therefore fulfilling the first objective of this study.

The *dotA*$^-$ mutants accumulated versicolorin A, an intermediate on the putative dothistromin biosynthetic pathway. Further disruptions of putative dothistromin biosynthetic genes will help elucidate the dothistromin biosynthetic pathway and confirm if the pathway proposed in this study is the pathway in the fungus. Confirmation of the function of *dotA* as a dothistromin biosynthetic gene provides evidence that the cluster of genes containing *dotA* on the λCGV1 clone could be part of a dothistromin biosynthetic cluster. *dotA*$^-$ mutants need to be rechecked to investigate if dothistromin is absent or if the mutants are just extremely low dothistromin producers.

The λBMKSA clone containing the partial PKS^{DOT} coding sequence, was further sequenced and a putative dothistromin cytochrome p450 mono-oxygenase gene was revealed and named *dcm1* along with the 5' coding sequence and promoter region of the pks^{dot} gene. The gene *dcm1* had high identity to AF and ST p450 cytochrome mono-oxygenase genes *cypX* and *stcB* respectively. The further sequencing of λBMKSA and discovery of a new putative dothistromin biosynthetic gene fulfilled the second objective of this study.

In an attempt to piece the λCGV1 and λBMKSA together in a single gene cluster, PCR amplification of genomic DNA using primers contained within different clones containing parts of the putative dothistromin cluster was tried but was unsuccessful (Laarakkers 1999)., (Astin 2000). Subsequently genomic *D. pini* digests were hybridised with probes from each clone to see if any hybridised to the same fragment. No probes hybridised to the same restriction fragment indicating that the λCGV1 and λBMKSA are not close in the *D. pini* genome. This does not indicate the clones are not in the same cluster as they could be as much as 40 kb apart and still be contained within the same cluster. Future work will include chromosome walking in an effort to find the intervening regions. Sequencing of new clones resulting from the chromosome walking will reveal whether the putative dothistromin genes are linked.

The order of genes within the cluster is not thought to be correlated to the action of the genes as homologs present in all three pathways are organised differently yet catalyse equivalent steps on the biosynthetic pathways. Neither the order of the homologs on λBMKSA or λCGV1 correspond to the order in either the *A. parasiticus* AF cluster or the *A. nidulans* ST cluster.

The differences in the regulatory binding sites between the putative dothistromin genes are correlated to the locus of the gene. The $TCGN_{11}CGA$ sequence has only been found in genes found in the clone λCGV1 while genes contained within λBMKSA have the same $TCGN_5CGA$ sequence found in AF and ST biosynthetic genes. The presence of two different regulatory motifs in putative genes from different clones indicates there could be

two separately regulated dothistromin biosynthetic clusters, or that the two clusters are involved in separate biosynthetic pathways, however, given the homologies between the genes found on the λBMKSA clone and the AF/ST genes, this seems unlikely. Disruption of the *pks*^{dot} gene on the λBMKSA will confirm if like *dotA*, it is involved in dothistromin biosynthesis. If *pks*^{dot} is a dothistromin biosynthetic gene then it is likely that other genes on λBMKSA are also involved in dothistromin biosynthesis.

D. pini is expected to have a regulatory protein homologous to AflR, however, nothing was identified either with probing the genome with a heterologous probe or degenerate PCR (Astin 2000). In regulation of this kind, the 'spacer' in the middle of the palindromic sequences (N=5 or N=11) is very important. The two different regulatory sites could mean however that there are two different regulatory proteins. Two regulatory proteins could regulate one pathway or the two clones could be from separate clusters, each with its own regulator.

The homology between genes on both clones to those from ST and AF biosynthesis suggests both clones are involved in polyketide biosynthesis. *D. pini* does not produce AF under the conditions tested but does produce the polyketide dothistromin. The gene contained within λCGV1, *dotA*, has been shown to be involved in dothistromin biosynthesis. Further disruption analysis of genes in these two clusters will confirm if they are both involved in dothistromin biosynthesis and disruptions of putative dothistromin genes such as *dcm1* and *pks*^{dot} and analysis of intermediates accumulated by the mutants will elucidate the biosynthetic pathway of dothistromin.

Dothistromin deficient mutants will allow the role of dothistromin in the pathogenicity of *D. pini* to be studied. Infecting *P. radiata* with the dothistromin deficient *D. pini* will allow pathogenicity of the mutants to be investigated and deduce the role of dothistromin in the disease.

Dothistromin research is able to contribute to understanding of the biosynthesis and function of the similar compounds AF and ST. Firstly in biosynthesis the biochemical

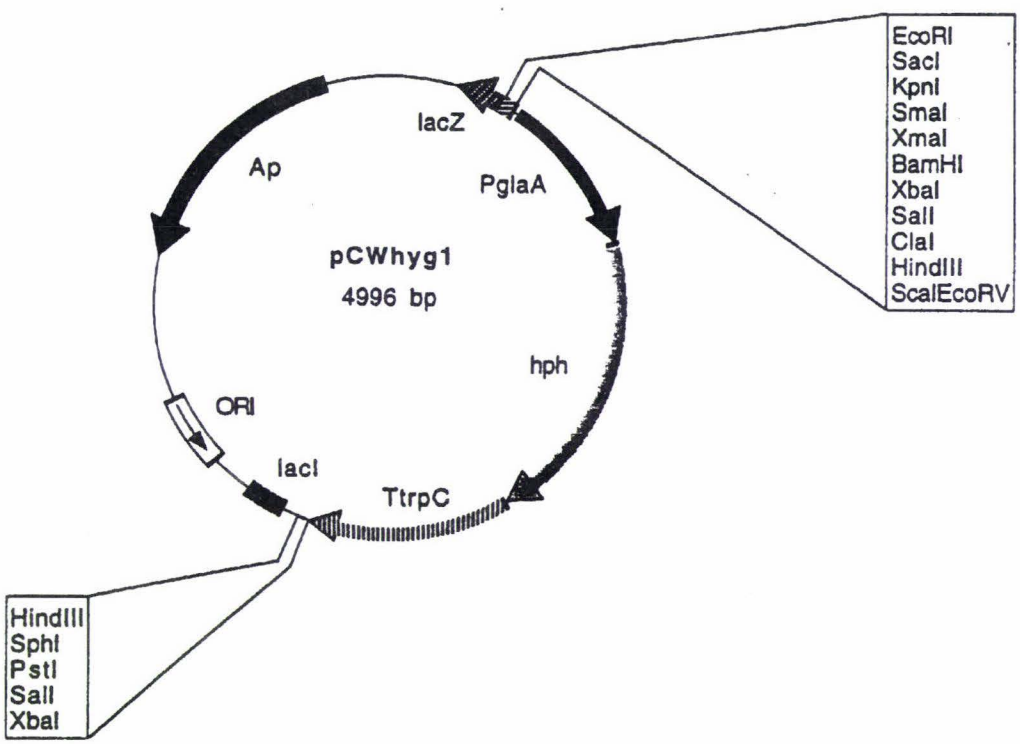
pathways for AF, ST and dothistromin diverge after versicolorin B, so homologous genes such as *stcB* and *cypX* must be in the pathway prior to divergence as the homolog *dcm*1 is present in *D. pini*. Secondly the biological role of metabolites AF and ST has not been determined while dothistromin has been proposed as the pathogenic factor in the disease *Dothistroma* needle blight caused by infection of *P. radiata* with *D. pini*.

It is not known why certain *Aspergillus* species produce the secondary metabolites AF and ST. It could be that the metabolites are artifacts of a time when they were needed for invasion of host plants or they could give *Aspergillus* an advantage over competitors. Deducing the role of dothistromin in pathogenicity may enhance the understanding of why *Aspergillus* produces the similar compounds AF and ST.

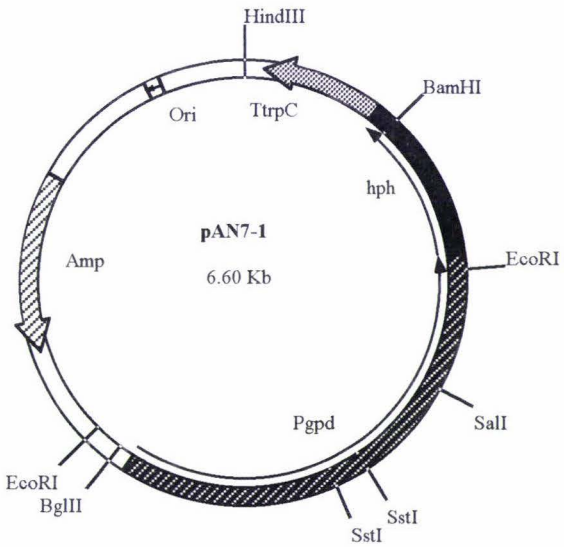
Appendices

APPENDIX 1: PLASMID MAPS

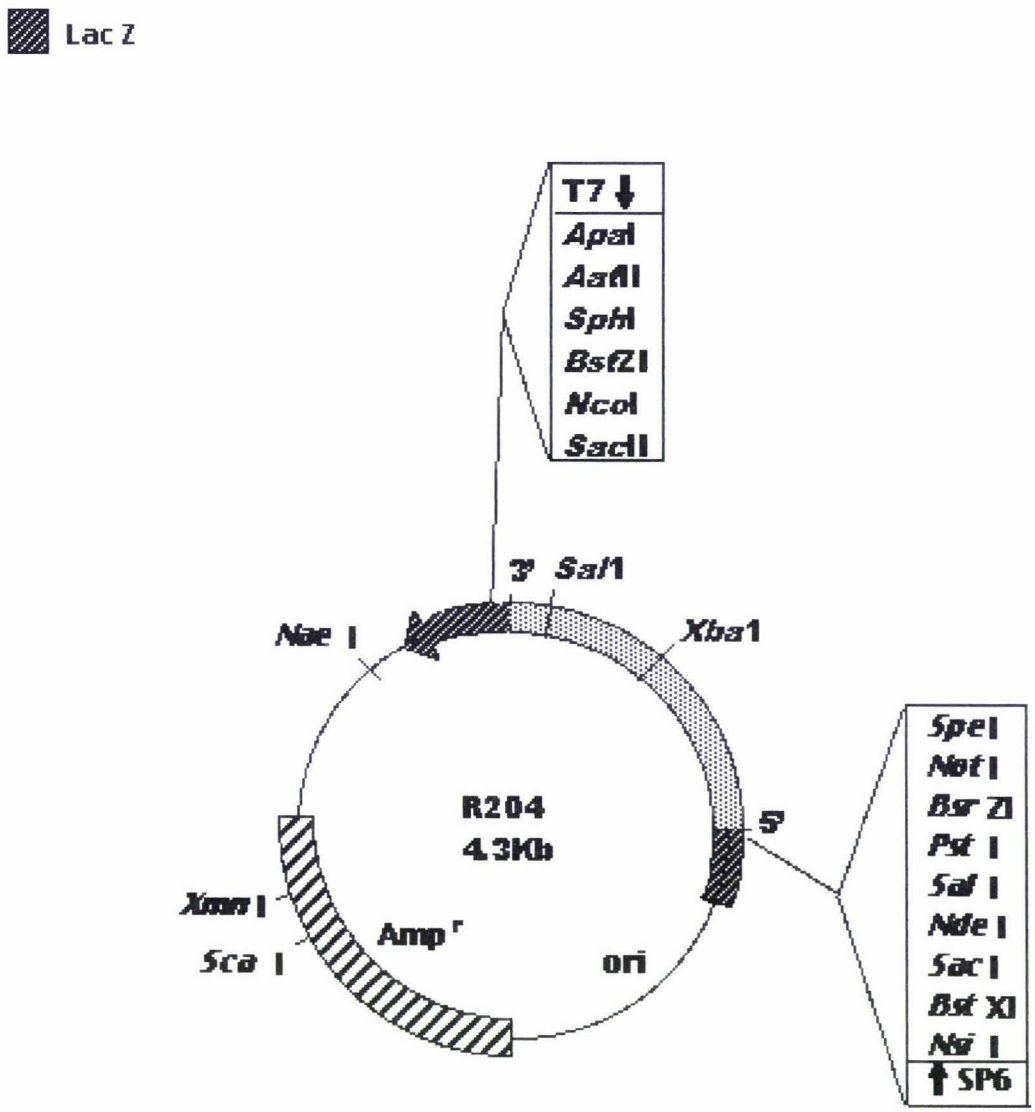
Appendix 1A: pCWhyg



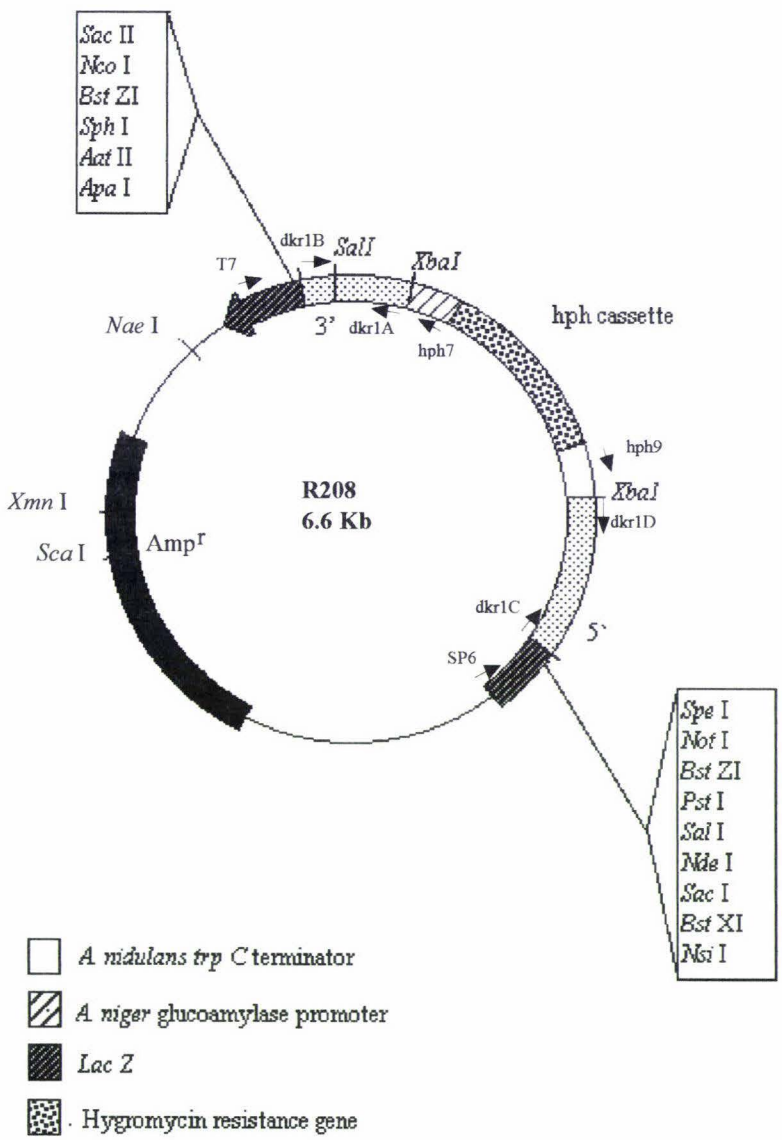
Appendix 1B: pAN7-1 (figure from Michelle McGill)



Appendix 1C: R204



Appendix 1D: R208



Appendix 2: Partial Sequence of λBMKSA Incorporating R181, R163, R156 and Overlaps.

NB: Nucleotides 1-510 have been sequenced on one strand only.

```
        10         20         30         40         50         60         70
         |          |          |          |          |          |          |
GAAGGACGTCAGTCNTTGCGAAGGCGATGTTNGAACATGCATTNTGCTGGAAATGTCAAGTCCNAGAGCC
CTTCCTGCAGTCAGNAACGCTTCCGCTACAANCTTGTACGTAANACGACCTTTACAGTTCAGGNTCTCGG

        80         90         100        110        120        130        140
         |          |          |          |          |          |          |
TACNGGGGGATTNTANNGCGTGTCGGGCACGGATCACAGATGGGTAATATGAATCGTGGGCCATGTCCGA
ATGNCCCCCTAANATNNCGCACAGCCCGTGCCTAGTGTCTACCCATTATACTTAGCACCCGGTACAGGCT

        150        160        170        180        190        200        210
         |          |          |          |          |          |          |
GCCGTCACGACTATTCGTGTGTTCTTCAACGGATGTCTGCCATCATGAATGGGCCTAAGTATGGCGCGCA
CGGCAGTGCTGATAAGCACACAAGAAGTTGCCTACAGACGGTAGTACTTACCCGGATTCATACCGCGCGT

        220        230        240        250        260        270        280
         |          |          |          |          |          |          |
TTGCCAGAGGATAAGGGGGGTTCTGCTGTCGATAGGAAGAAGCGACGTTGCACACCAACAACACCGACAA
AACGGTCTCCTATTCCCCCCAAGACGACAGCTATCCTTCTTCGCTGCAACGTGTGGTTGTTGTGGCTGTT

        290        300        310        320        330        340        350
         |          |          |          |   R181fwdsch ->    |          |
TCCTCCATGCATGTGCACATGTCCCCGGAGTCCGGCGATGCACAATGCGACGGAATGCTGCCGCGAGGAG
AGGAGGTACGTACACGTGTACAGGGGCCTCAGGCCGCTACGTGTTACGCTGCCTTACGACGGCGCTCCTC

        360        370        380        390        400        410        420
         |          |          |          |          |          |          |
GGCTGTACTATGCATGCATGACTGAGTATACCAGCCAGATGGGGTACGACTTGGCAAAAGATGCGGTTCT
CCGACATGATACGTACGTACTGACTCATATGGTCGGTCTACCCCATGCTGAACCGTTTTCTACGCCAAGA

        430        440        450        460        470        480        490
         |          |          |          |          |          |          |
NATGGTCGGGGTGTGTGNNTGTTGTGATGAAGAGTNAGGTGACCACGGGTGAAGAAGACTGCAGACGAAG
NTACCAGCCCCACACACNNACAACACTACTTCTCANTCCACTGGTGCCCACTTCTTCTGACGTCTGCTTC

        500        510        520        530        540        550        560
         |          |          |          |          |          |          |
TGGCAGTGGAAGTGGGTGAGAAAGATGATGTAAAACAGTCCATCAATATCGNGTGCAGATACCCCTAAAA
ACCGTCACCTTCACCCACTCTTTCTACTACATTTTGTCAGGTAGTTATAGCNCACGTCTATGGGGATTTT
<- R181revch

        570        580        590        600        610        620        630
         |          |          |          |          |          |          |
CTTGATTGACTCTGCAACAATGTGTGTTTCGTTTTCCCCGTTTACACACGACACGGCCTCGACCTTTACT
GAACTAACTGAGACGTTGTTACACACAAAGCAAAAGGGGCAAATGTGTGCTGTGCCGGAGCTGGAAATGA

        640        650        660        670        680        690        700
         |          |          |          |          |          |          |
TCACCCAATGCACAACAACATCCATTCCTTCATGCTCATGCCTTCATCGTCACTTCGCACCGCCTGTCTC
AGTGGGTTACGTGTTGTTGTAGGTAAGGAAGTACGAGTACGGAAGTAGCAGTGAAGCGTGGCGGACAGAG

        710        720        730        740        750        760        770
         |          |          |          |          |          |          |
TCGGTACACCGGCAACGAAACTGTCCACCACCGCCATGCTCTCAGGCGTCGCCGACTTGGCCAGCTTGAC
AGCCATGTGGCCGTTGCTTTGACAGGTGGTGGCGGTACGAGAGTCCGCAGCGGCTGAACCGGTCGAACTG
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         780        790        800        810        820        830        840
         |          |          |          |          |          |          |
TCCGCGCAGCTCTCGGAAGAAGTGAGCAACGGCAAGACGCATTTCCAACCTGCCCAGATGAATGCCCAAA
AGGCGCGTCGAGAGCCTTCTTCACTCGTTGCCGTTCTGCGTAAAGGTTGGACGGGTCTACTTACGGGTTT

         850        860        870        880        890        900        910
         |          |          |          |          |          |          |
CACTGCCTGGAACCTTGGCCGAACGGGTTGAACGCCATCTTCTGCTTCTCACTGAATTCGAGCCTGTTCT
GTGACGGACCTTGGAACCGGCTTGCCCAACTTGCGGTAGAAGACGAAGAGTGACTTAAGCTCGGACAAGA
                                                    |
                                                  EcoRI

         920        930        940        950        960        970        980
         |          |          |          |          |          |          |
CGGGCAGGAAGCGCGTGTGGTCGAATCTGTCGCAACATTAGCATCTCCCCCGAGCATCCACCACACATAC
GCCCGTCCTTCGCGCACACCAGCTTAGACAGCGTTGTAATCGTAGAGGGGGCTCGTAGGTGGTGTGTATG

         990        1000       1010       1020       1030       1040       1050
         |          |          |          |          |          |          |
ACAACTCACGTCTGTGCCTCCTTCCACGTGGCACCATCCGTGTGCAAAGTCCAATTCTGCGTGTCGACGA
TGTTGAGTGCAGACACGGAGGAAGGTGCACCGTGGTAGGCACACGTTTCAGGTTAAGACGCACAGCTGCT

         1060       1070       1080       1090       1100       1110       1120
         |          |          |          |          |          |          |
CAGTGCCTTCGGGGATGTAGTATCCACCAAGGTTGGCACCACCAGCTGGCGGGCTGCGTGGAAGTCCTCC
GTCACGGAAGCCCCTACATCATAGGTGGTTCCAACCGTGGTGGTCGACCGCCCGACGCACCTTCAGGAGG

         1130       1140       1150       1160       1170       1180       1190
         |          |          |          |          |          |          |
GGGAGCTGCACCGTACAGTCGCATGCTTTCGTCAATGATGGCGTTCATCAGCGGCAGTCCCTCACATACG
CCCTCGACGTGGCATGTCAGCGTACGAAAGCAGTTACTACCGCAAGTAGTCGCCGTCAGGGAGTGTATGC

         1200       1210       1220       1230       1240       1250       1260
         |          |          |          |          |          |          |
GTATCCGTCACTTCTCCATCGATCGACGCGACTTCCTCCTCCAGCTGCTTCTGGAGCTCAGGTCGGCTGA
CATAGGCAGTGAAGAGGTAGCTAGCTGCGCTGAAGGAGGAGGTCGACGAAGACCTCGAGTCCAGCCGACT

         1270       1280       1290       1300       1310       1320       1330
         |          |          |          |          |          |          |
GAACGAGGTAGATCAGGAACGTCAGCGAAATGGCAGTCGGGTCGGAGCCGGCGAGCAACAGAGCACCGGC
CTTGCTCCATCTAGTCCTTGCAGTCGCTTTACCGTCAGCCCAGCCTCGGCCGCTCGTTGTCTCGTGGCCG

         1340       1350       1360       1370       1380       1390       1400
dmo1R -> |          |          |          |          |          |          |
GTCAGTGATGATGTCCGTATCAAGTCAACGTCGCAAGCATCGCCCTCGCCCTCCTGCAACGCCTTTGCGA
CAGTCACTACTACAGGCATAGTTCAGTTGCAGCGTTCGTAGCGGGAGCGGGAGGACGTTGCGGAAACGCT
 <- dmoC3

         1410       1420       1430       1440       1450       1460       1470
         |          |          |          |          |          |          |
ACAAGTTCTGGTTGAACTCGCCAGCCTCCTTGTCCTGACGAGCGCTCTTCACCACGCCAGCACCGGCCTT
TGTTCAAGACCAACTTGAGCGGTCGGAGGAACAGGACTGCTCGCGAGAAGTGGTGCGGTCGTGGCCGGAA

         1480       1490       1500       1510       1520       1530       1540
         |          |          |          |          |          |          |
GAACATTTTCTCCTGCGAGTAGAAAATGTCGTTCATGCGCGTGTTGACCTTTCCGAGTACCCGACCGACG
CTTGTAAAAGAGGACGCTCATCTTTTACAGCAAGTACGCGCACAACTGGAAAGGCTCATGGGCTGGCTGC

         1550       1560       1570       1580       1590       1600       1610
         |          |          |          |          |          |          |
TAGTAAAGAGGGGGAATGAACATCTTGAGGAGGTGTGCGAGGTCGCCTTTACGCTTCTCAAGCATGAGCA
ATCATTTCTCCCCCTTACTTGTAGAACTCCTCCACACGCTCCAGCGGAAATGCGAAGAGTTCGTACTCGT
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         1620       1630       1640       1650       1660       1670       1680
         |          |          |          |          |          |          |
CGAATGGATCTTTGATACCCTTCTCGACAGTTCCGTGTCCGCCATTGAACGTCAGGCGGCAGACAATCTC
GCTTACCTAGAAACTATGGGAAGAGCTGTCAAGGCACAGGCGGTAACTTGCAGTCCGCCGTCTGTTAGAG

         1690       1700       1710       1720       1730       1740       1750
         |          |          |          |          |          |          |
GTTGGCCATCAGCGTCCACCAGCCCATGAGCTCGGTGTCGCCATTGGCAGCCTCTTCCTTCATCTTCTGC
CAACCGGTAGTCGCAGGTGGTCGGGTACTCGAGCCACAGCGGTAACCGTCGGAGAAGGAAGTAGAAGACG
                   <- dmoCB

         1760       1770       1780       1790       1800       1810       1820
         |          |          |          |          |          |          |
ACGGCCATGCTGATGGTCTTGTTGATCGTGCTTTCCCAATTCTTTCTCAGCTCGACAAGAGTAAAGCCCT
TGCCGGTACGACTACCAGAACAACTAGCACGAAAGGGTTAAGAAAGAGTCGAGCTGTTCTCATTTCGGGA

         1830       1840       1850       1860       1870       1880       1890
         |          |          dmoR2 ->     |          |          |          |
TGGCATACAAGCTTGCGGCGCTGGCTGTGCTTCTTCTGGTCGCGAAAGTTGAAGATGTTGTCAACCGGGC
ACCGTATGTTCGAACGCCGCGACCGACACGAAGAAGACCAGCGCTTTCAACTTCTACAACAGTTGGCCCG

         1900       1910       1920       1930       1940       1950       1960
         |          |          |          |          |          |          |
CTGGAGAAAGGAGACGATAGAACGGAGCTTTTGTGAACACTGTACCCATGCGGTGCACCTCCCGTGCCGC
GACCTCTTTCCTCTGCTATCTTGCCTCGAAAACACTTGTGACATGGGTACGCCACGTGGAGGGCACGGCG

         1970       1980       1990       2000       2010       2020       2030
         |          |          |          |          |          |          |
CGCTGGATCTGCAACATCCACCTCCTTGGGACCGATCCTCACCATGGGGCCATACTTTTGGTGCAGTGAA
GCGACCTAGACGTTGTAGGTGGAGGAACCCTGGCTAGGAGTGGTACCCCGGTATGAAAACCACGTCACTT

         2040       2050       2060       2070       2080       2090       2100
         |          |          |          |          |          |          |
TCAACATAGTAGATGCGGTTGCCAGCGAAGACGGAGTACGTCAGGCGCAAGTCTGTCAACTTGGCATACC
AGTTGTATCATCTACGCCAACGGTCGCTTCTGCCTCATGCAGTCCGCGTTCAGACAGTTGAACCGTATGG

         2110       2120       2130       2140       2150       2160       2170
         |          |          |          |          |          |          |
ATGGTCCTGGAATCTTGCTCAGAGGAGAAAAGTATGCTGTCGTGATGATCTGCAACATCTATTAGCAGCG
TACCAGGACCTTAGAACGAGTCTCCTCTTTTCATACGACAGCACTACTAGACGTTGTAGATAATCGTCGC

         2180       2190       2200       2210       2220       2230       2240
         |          |          |          |          |          |          |
TTTCCACCGCGAAAATTGACGTCTACTCACAGAAACAATGTTATACAGAACAAACGCAGCAGCGACAAGT
AAAGGTGGCGCTTTTAACTGCAGATGAGTGTCTTTGTTACAATATGTCTTGTTTGCGTCGTCGCTGTTCA

         2250       2260       2270       2280       2290       2300       2310
         |          |          |          |          |          |          |
GCCAGCGAAAACGGCAATGGAGCACCAGCAGTGGCGTCCATAATCCACTTGTAGAGCTCTCCTGCCATGG
CGGTCGCTTTTGCCGTTACCTCGTGGTCGTCACCGCAGGTATTAGGTGAACATCTCGAGAGGACGGTACC
                                                 <- dmoCA

         2320       2330       2340       2350       2360       2370       2380
         |          |          |          |          |          |          |
TGTCGGAGTGGCAGTGAAGACCAGCAGACGTTGAAAGAGAATGACAAACGGCGTCAAGAACAGTAAGTAA
ACAGCCTCACCGTCACTTCTGGTCGTCTGCAACTTTCTCTTACTGTTTGCCGCAGTTCTTGTCATTCATT

         2390       2400       2410       2420       2430       2440       2450
         |          |          | dmoR3 ->|          |          |          |
CTTGTCAATGCTATCATGCAATCCGTTGATACTACACAAGCCTCGCAGAACGACTTGTCATCAACAAAGC
GAACAGTTACGATAGTACGTTAGGCAACTATGATGTGTTCGGAGCGTCTTGCTGAACAGTAGTTGTTTCG
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         2460       2470       2480       2490       2500       2510       2520
         |          |          |          |          |          |          |
GGTATTCCGCACGCTCTGAGTGATCCTTATGTCGGTTTGCGATACTTCGGACTTCAGGCTCGGTCAAGTG
CCATAAGGCGTGCGAGACTCACTAGGAATACAGCCAAACGCTATGAAGCCTGAAGTCCGAGCCAGTTCAC
                                                <- dmoF4

         2530       2540       2550       2560       2570       2580       2590
         |          |          |          |          |          |          |
AATCCAGCCCGAAACAGCCAGCGCAAGCCCACGGCAGTACCCGATTGGATCGATTGATCGATACCGCGCA
TTAGGTCGGGCTTTGTCGGTCGCGTTCGGGTGCCGTCATGGGCTAACCTAGCTAACTAGCTATGGCGCGT

         2600       2610       2620       2630       2640       2650       2660
         |          |          |          |          |          |          |
AACTGCTGCTTGCCGTCGGCCTGCACGTGTATGCCATGCGAATGCGCTAACTCTGCCTCAGCTATCGTAA
TTGACGACGAACGGCAGCCGGACGTGCACATACGGTACGCTTACGCGATTGAGACGGAGTCGATAGCATT

         2670       2680       2690       2700       2710       2720       2730
         |          |          |          |          |          |          |
TCGCCAAACCCTTCGGCCTGGCCAGCCGTCCTCACAGTGGCCCGACTGTTGCTGGCGGACAGCGACCGTA
AGCGGTTTGGGAAGCCGGACCGGTCGGCAGGAGTGTCACCGGGCTGACAACGACCGCCTGTCGCTGGCAT

         2740       2750       2760       2770       2780       2790       2800
         |          |          |          |          |          | PKSCA ->|
TCTCGCAGGCAAGACCCGAAGACAAATTGACTTTGATACGCAGGCCCAGTACACTTTCCTCGCTGTCTTC
AGAGCGTCCGTTCTGGGCTTCTGTTTAACTGAAACTATGCGTCCGGGTCATGTGAAAGGAGCGACAGAAG

         2810       2820       2830       2840       2850       2860       2870
         |          |          |          |          |          |          |
CTCGACCTGTTGACTGCCATCGATCCCAGCACCGCTCACCATACAGGCTGTCCCCTGTCTCTACACCTAC
GAGCTGGACAACTGACGGTAGCTAGGGTCGTGGCGAGTGGTATGTCCGACAGGGGACAGAGATGTGGATG

         2880       2890       2900       2910       2920       2930       2940
         |          |          |          |          |          |          |
ACTCATTCTTTCCAGGTGTGCTTTGCGCCTCGTTGATGACACACTAATTTCGATACTCAGGCGACTTTCT
TGAGTAAGAAAGGTCCACACGAAACGCGGAGCAACTACTGTGTGATTAAAGCTATGAGTCCGCTGAAAGA

         2950       2960       2970       2980       2990       3000       3010
         |          |          |          |          |          |          |
TCGCCATAACCTCCCCACACAGTCAATATGACACACTCCAACGCAACGAGGGTACTGGTCTTTGGTGACC
AGCGGTATTGGAGGGGTGTGTCAGTTATACTGTGTGAGGTTGCGTTGCTCCCATGACCAGAAACCACTGG

         3020       3030       3040       3050       3060       3070       3080
         |          |          |          |          |          |          |
AGACCTATGACTTTGTGCCTAAGCTTCGAGAACTGTTCCACGTCAAGGATAACCCGATCTTGACGGCTTT
TCTGGATACTGAAACACGGATTCGAAGCTCTTGACAAGGTGCAGTTCCTATTGGGCTAGAACTGCCGAAA

         3090       3100       3110       3120       3130       3140       3150
         |          |          |          |          |          |          |
CCTGGAACAGTCTCATTATGTCGTGTAAGTCGTACTGATCGATCGGACGCAGACCGTTGCTAAGAATTGC
GGACCTTGTCAGAGTAATACAGCACATTCAGCATGACTAGCTAGCCTGCGTCTGGCAACGATTCTTAACG
      <- dmoF3

         3160       3170       3180       3190       3200       3210       3220
         |          |          |          |          |          |          |
CAGCCGAGCACAGATGATCCAGACACTGCCTCCGGCGGAGCACAAGGCAGCTCGAACCTTCGACTTAGCA
GTCGGCTCGTGTCTACTAGGTCTGTGACGGAGGCCGCCTCGTGTTCCGTCGAGCTTGGAAGCTGAATCGT

         3230       3240       3250       3260       3270       3280       3290
         |PKSCB2 ->|          |          |          |          |          |
GATATGCTGAAGAAGTATGTCGCCGGCAAGCTGAACCCTGCCTTCCAGACGGCCCTCAGCTGCATCACAC
CTATACGACTTCTTCATACAGCGGCCGTTCGACTTGGGACGGAAGGTCTGCCGGGAGTCGACGTAGTGTG
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         3300       3310       3320       3330       3340       3350       3360
         |          |          |          |          |          |          |
AACTCGGTGTGTTCATGCGAGAGTTCCATGACTTCACCAAGCCATATCCACGACACGATAGCAGCTACGT
TTGAGCCACACAAGTACGCTCTCAAGGTACTGAAGTGGTTCGGTATAGGTGCTGTGCTATCGTCGATGCA

         3370       3380       3390       3400       3410       3420       3430
         |          |          |          |          |          |          |
GTTGGGTATCTGCACCGGCTCGCTTGCCGCTGCGGCCGTCAGCTCCAGCAACTCTTTATCCGAGCTTCTG
CAACCCATAGACGTGGCCGAGCGAACGGCGACGCCGGCAGTCGAGGTCGTTGAGAAATAGGCTCGAAGAC

         3440       3450       3460       3470       3480       3490       3500
         |          |          |          |          |          |          |
CCTATTGCCGTGCAAACGGCTTTGATCGCCTTTCGCCTCGGTCTGTGCGTCACAGACATGCGAGATCGTC
GGATAACGGCACGTTTGCCGAAACTAGCGGAAAGCGGAGCCAGACACGCAGTGTCTGTACGCTCTAGCAG

         3510       3520       3530       3540       3550       3560       3570
         |          |          |          |          |          |          |
TCGAAAGCTCGGAAGAAGACCGCACCCAGCCTTGGTCGGTAGTTCTGTTCGACACAGACGAGCAGACTGT
AGCTTTCGAGCCTTCTTCTGGCGTGGGTCGGAACCAGCCATCAAGACAAGCTGTGTCTGCTCGTCTGACA

         3580       3590       3600       3610       3620       3630       3640
         |          |          |          |          |          |          |
TACCAAGGCCATCAAAGACTTCTGCACATCCAACGTCCTCCCGAAGACCAAGCAGCCCTGGATTACCTCC
ATGGTTCCGGTAGTTTCTGAAGACGTGTAGGTTGCAGGAGGGCTTCTGGTTCGTCGGGACCTAATGGAGG

         3650       3660       3670       3680       3690       3700       3710
         |          |          |          |          |          |          |
GCGTCATCGAAGACAATCTCCATCAGCGGAGCACCACGTGTGCTGAAGAAGTTGTCGCAAGAGCCTGCGC
CGCAGTAGCTTCTGTTAGAGGTAGTCGCCTCGTGGTGCACACGACTTCTTCAACAGCGTTCTCGGACGCG
 <- dmoF2

         3720       3730       3740       3750       3760       3770       3780
         |          |          |          |          |          |          |
TCAAGGACAAAAAGACCAGACAGATCCCGATTTATGTCCCAGCACACAATTCTGCCCTCTTCACCCCCGA
AGTTCCTGTTTTTCTGGTCTGTCTAGGGCTAAATACAGGGTCGTGTGTTAAGACGGGAGAAGTGGGGGCT

         3790       3800       3810       3820       3830       3840       3850
 PKSC3 ->|          |          |          |          |          |          |
AGATGTCAAGTCTATCCTTGAGACTACCCCTGTCGACACTTGGAGCAACTACCCTACCAAGCTTCCATTC
TCTACAGTTCAGATAGGAACTCTGATGGGGACAGCTGTGAACCTCGTTGATGGGATGGTTCGAAGGTAAG

         3860       3870       3880       3890       3900       3910       3920
         |          |          |          |          |          |          |
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TAGAGCTCGCAAAGACCGTTCTACCGAACCCGTCTGTTGATGGAACGTCATTAGGTAGAGCGGGAGTTAG

         3930       3940       3950       3960       3970       3980       3990
         |          |          |          |          |          |          |
AGTGCCTGCTCGAGAGCATTGGCTGGGGTAAGGTCGAGACTGAGCTCCCAAGGCTCCTCAAGTCTCGCGG
TCACGGACGAGCTCTCGTAACCGACCCCATTCCAGCTCTGACTCGAGGGTTCCGAGGAGTTCAGAGCGCC
 <- dmo1F

         4000       4010       4020       4030       4040       4050       4060
         |          |          |          |          |          |          |
CGCAGAAAATGTGCTCATCACGCCAATCACCACCTCTGCCGACCGTGCTCTGTCGGCTGCACTCAGCCCA
GCGTCTTTTACACGAGTAGTGCGGTTAGTGGTGGAGACGGCTGGCACGAGACAGCCGACGTGAGTCGGGT

         4070       4080       4090       4100       4110       4120       4130
         |          |          |          |          |          |          |
ACGATCTCCAATATCGAGGTCGAGAAGCCCACAATCAACGAATCTTTCGCCCATAGACCTGGTTCGGGAA
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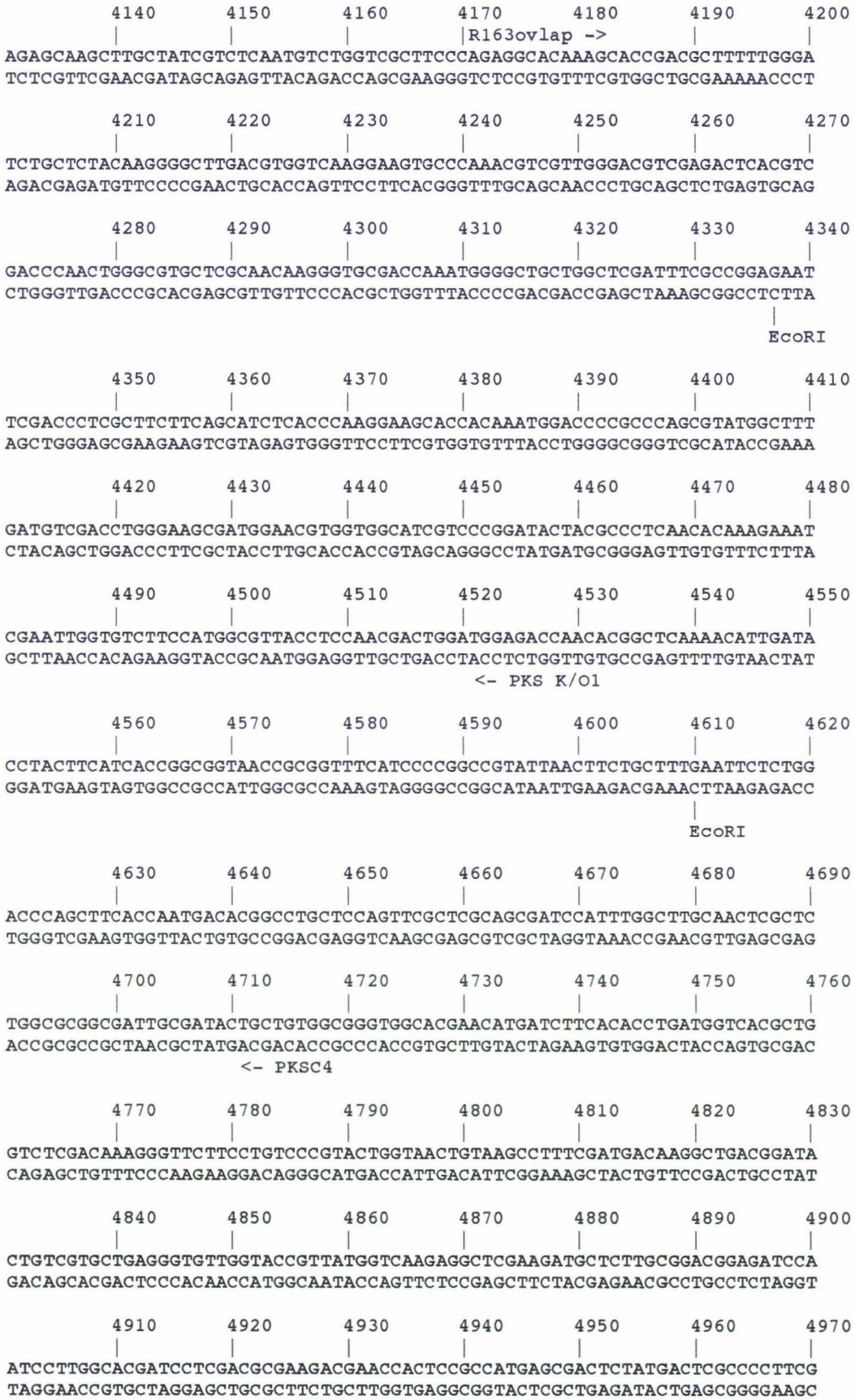
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4210 4220 4230 4240 4250 4260 4270
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4280 4290 4300 4310 4320 4330 4340
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EcoRI
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<- PKS K/O1
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EcoRI
4630 4640 4650 4660 4670 4680 4690
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4700 4710 4720 4730 4740 4750 4760
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<- PKSC4
4770 4780 4790 4800 4810 4820 4830
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4910 4920 4930 4940 4950 4960 4970
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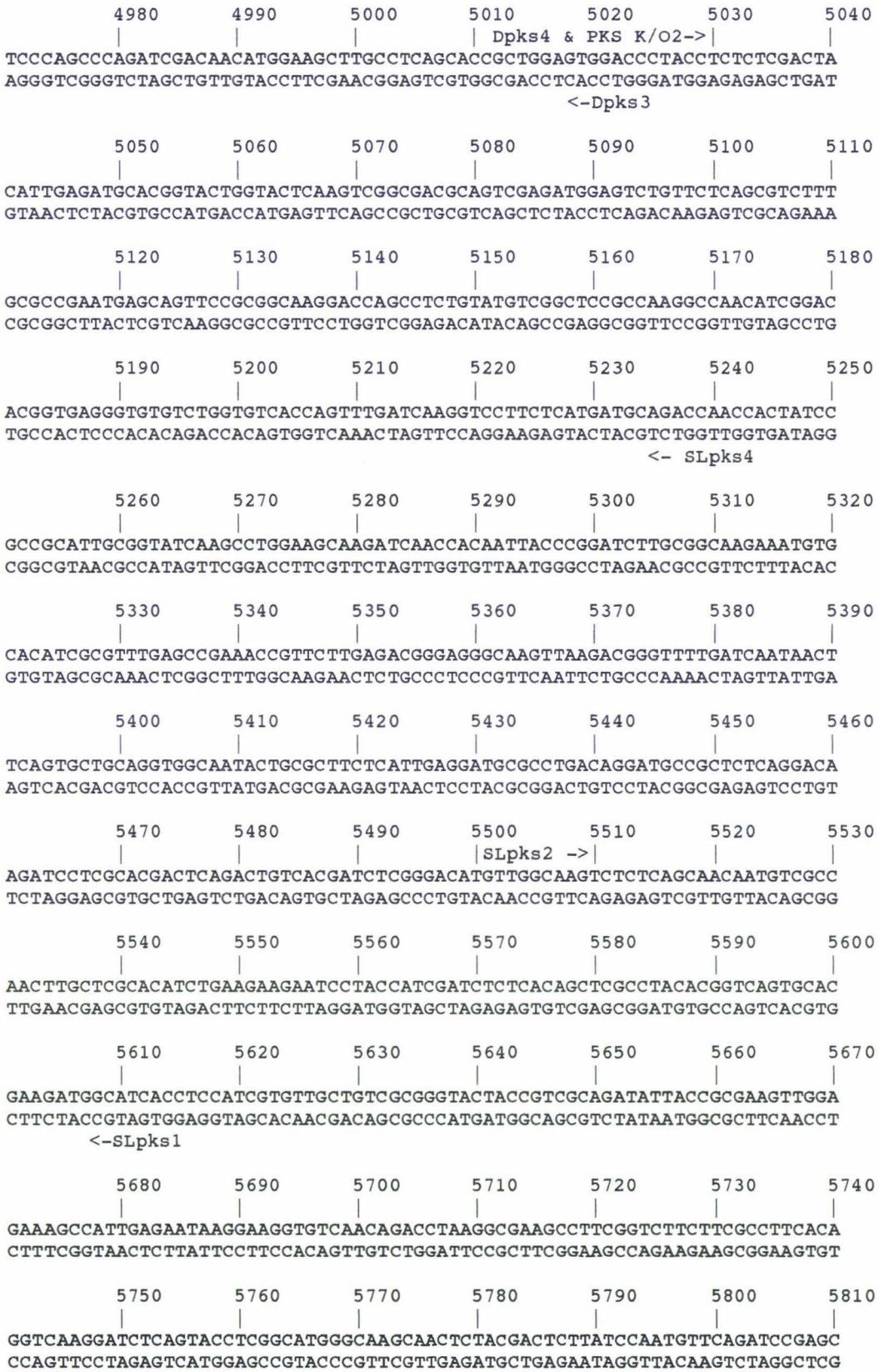
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<-Dpks3
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5120 5130 5140 5150 5160 5170 5180
GCGCCGAATGAGCAGTTCCGCGGCAAGGACCAGCCTCTGTATGTCGGCTCCGCCAAGGCCAACATCGGAC
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5190 5200 5210 5220 5230 5240 5250
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<- SLpks4
5260 5270 5280 5290 5300 5310 5320
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5330 5340 5350 5360 5370 5380 5390
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5400 5410 5420 5430 5440 5450 5460
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5470 5480 5490 5500 5510 5520 5530
SLpks2 ->
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5540 5550 5560 5570 5580 5590 5600
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5610 5620 5630 5640 5650 5660 5670
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<-SLpks1
5680 5690 5700 5710 5720 5730 5740
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5750 5760 5770 5780 5790 5800 5810
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```
         5820       5830       5840       5850       5860       5870       5880
         |          |          |          |          |          |          |
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AAGTTCCGATGCTAGCGAACCGTGTTAGCGTTCCGAAGGGTTCGAAACGTGTGTAGAAGTGGCTCTGCTT

         5890       5900       5910       5920       5930       5940       5950
         |          |          |          |SLpks3 ->|          |          |
GGGAGATGTTGAACAGAATCTTCCAGTGGTCGTGCAGCTTGCTATTACATGCTTGCAAATGGCTCTCTTC
CCCTCTACAACTTGTCTTAGAAGGTCACCAGCACGTCGAACGATAATGTACGAACGTTTACCGAGAGAAG
                                                <- Dpks5

         5960       5970       5980       5990       6000       6010       6020
         |          |          |          |          |          |          |
AACCTCGTCACCTCCTTCGGAATCAAGGCCTCTGCCGTTGTCGGCCACTCGCTGGGCGAGTACGCTGCGC
TTGGAGCAGTGGAGGAAGCCTTAGTTCCGGAGACGGCAACAGCCGGTGAGCGACCCGCTCATGCGACGCG

         6030       6040       6050       6060       6070       6080       6090
         |          |          |          |          |          |          |
TGTATGCAGCTGGTGTGTTGAGTGCCAGCGACACGATCTACCTGGTCGGCAAACGTGCCGAGCTTCTCCA
ACATACGTCGACCACACAACTCACGGTCGCTGTGCTAGATGGACCAGCCGTTTGCACGGCTCGAAGAGGT

         6100       6110       6120       6130       6140       6150       6160
         |          |          |          |          |          |          |
GGATCATTGCCAGAGGGGTACGCATGCGATGCTTGCGTGCAAGGCGAGTGAGTGGAGTCTCGCCGAGATC
CCTAGTAACGGTCTCCCCATGCGTACGCTACGAACGCACGTTCCGCTCACTCACCTCAGAGCGGCTCTAG

         6170       6180       6190       6200       6210       6220       6230
         |          |          |          |          |          |          |
ACGGCGGGCAAGAATGTCGAAGTCGCATGCGTTAATGGGCCTGAAGACACTGTCCTCTCCGGCACTGTCG
TGCCGCCCGTTCTTACAGCTTCAGCGTACGCAATTACCCGGACTTCTGTGACAGGAGAGGCCGTGACAGC

         6240       6250       6260       6270       6280       6290       6300
         |          |          |          |          |          |          |
AGGAAATTGGAGAGGTGCAGAAGACACTCATTGCGAAGAGCATCAAGGCTACACTCTTGAAGTTGCCCTT
TCCTTTAACCTCTCCACGTCTTCTGTGAGTAACGCTTCTCGTAGTTCCGATGTGAGAACTTCAACGGGAA

         6310       6320       6330       6340       6350       6360       6370
         |          |          |          |          |          |          |
CGCGTTTCATTCGGCGCAGGTACAACCTATCCTCCGAGGACGTTCTGAAGAACTTGCGGCTGGAGCTACT
GCGCAAAGTAAGCCGCGTCCATGTTGGATAGGAGGCTCCTGCAAGACTTCTTGAACGCCGACCTCGATGA

         6380       6390       6400       6410       6420       6430       6440
         |          |          |          |          |          |          |
TTTGAGAAGCCCAAGCTTGCGGTCATTTCCCCGCTACTGGGCAGTGTGGTCGACGACGAAGGAGTCGTTG
AAACTCTTCGGGTTCGAACGCCAGTAAAGGGGCGATGACCCGTCACACCAGCTGCTGCTTCCTCAGCAAC

         6450       6460       6470       6480       6490       6500       6510
         |          |          |          |          |          |          |
GACCCAACTACCTTGCACGCCACTGCCGTGAGGCGGTCGGAATGGTCAAAGCCCTCGGAGTGGCGAAGGA
CTGGGTTGATGGAACGTGCGGTGACGGCACTCCGCCAGCCTTACCAGTTTCGGGAGCCTCACCGCTTCCT

         6520       6530       6540       6550       6560       6570       6580
    Dpks2 ->        |          |          |          |          |          |
GAAGGGTATAATCAACGAGAAGACCTTCGTCATTGAGATTGGTCCTAAGCCGCTTCTCTGCGGAATGATC
CTTCCCATATTAGTTGCTCTTCTGGAAGCAGTAACTCTAACCAGGATTCGGCGAAGAGACGCCTTACTAG
       <- Dpks1

         6590       6600       6610       6620       6630       6640       6650
         |          |          |          |          |          |          |
AAGAACATACTCGGCCAGAACATCGTAGCCTTGCCTACGTTGAAGGACAAGGGTCCAGACGTCTGGCAGA
TTCTTGTATGAGCCGGTCTTGTAGCATCGGAACGGATGCAACTTCCTGTTCCCAGGTCTGCAGACCGTCT
```

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         6660       6670       6680       6690       6700       6710       6720
         |          |          |          |          |          |          |
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TGGAGAGCTTGTAGAAGTGCTGCGAGATGTGGCCACCAAATCTGTAGTTGACCTGACGGAAGGTGCGGGG

         6730       6740       6750       6760       6770       6780       6790
         |          |          |          |          |          |          |
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GAAGCTCGGGCGCTTCTTCCAGGACGTTGAAGGACTAATACCGACCCTAGAGTTCCTAATGAAGTAGGTC

         6800       6810       6820       6830       6840       6850       6860
         |          |          |          |          |          |          |
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         6870       6880       6890       6900       6910       6920       6930
         |          |          |          |          |PKSSP6C->|          |
ATAACACTTCGCACTACTGTCCTGGCAAACACACCTTCGCTGAGAATGTTGTCGTTCCTGGTGGGGCTCA
TATTGTGAAGCGTGATGACAGGACCGTTTGTGTGGAAGCGACTCTTACAACAGCAAGGACCACCCCGAGT

         6940       6950       6960       6970       6980       6990       7000
         |          |          |          |          |          |          |
GAAGGCCGTTCAGGAAGCACCTGCGGCGAAGACAGAGACGAAGAAGATGTCGAAGCTGGATCCTACCAAG
CTTCCGGCAAGTCCTTCGTGGACGCCGCTTCTGTCTCTGCTTCTTCTACAGCTTCGACCTAGGATGGTTC
                                                          |
                                                        BamHI

         7010       7020       7030       7040       7050       7060       7070
         |          |          |          |          |          |          |
GAGGCGTATCCGGGCATTCCGCTCACCACGACCGTGCACAAGGTCATTGAAGAGAAGACGGAGCCTCTGG
CTCCGCATAGGCCCGTAAGGCGAGTGGTGCTGGCACGTGTTCCAGTAACTTCTCTTCTGCCTCGGAGACC

         7080       7090       7100       7110       7120       7130       7140
         |          |          |          |          |          |          |
GAGCGCAGTTCACGGTCGAGACGGATATCTCCCGCAAGGATGTCAACAGCATCGCTCAAGGTCACACTGT
CTCGCGTCAAGTGCCAGCTCTGCCTATAGAGGGCGTTCCTACAGTTGTCGTAGCGAGTTCCAGTGTGACA

         7150       7160       7170       7180       7190       7200       7210
         |          |          |          |          |          |          |
TGACAGCATTCCCCTCTGCACGCCATCATTCTATGCGGATATTGCGCTTCAGGTTGGCAAATACGCCATG
ACTGTCGTAAGGGGAGACGTGCGGTAGTAAGATACGCCTATAACGCGAAGTCCAACCGTTTATGCGGTAC

         7220       7230       7240       7250       7260       7270
         |          |          |          |          |          |
GACCGCATCCGTGCTGGACATCCCGGTGCCGGTGCTATTGATGGAAGGGTCGATGTTACGGATCGAG
CTGGCGTAGGCACGACCTGTAGGGCCACGGCCACGATAACTACCTTCCCAGCTACAATGCCTAGCTC
```

APPENDIX 3: Flanking Regions of *dotA* used for the *dotA* Disruption Vector

Flanking regions for the disruption vector were designed in order to replace the NADPH binding site with the *hph* cassette while still working within the following constraints: flanking regions should not include or interfere with genes at close proximity to the site of recombination, and, flanking regions should be amplified using primer positions suggested by the primer design program.

Primers: dkr1C

dkr1D

dkr1A

dkr1B

putative NADPH binding site.

Arrows show direction of transcription.

GCTACACATT CGTGTCTATC ATGAATCTTC GTCGCTCAGC TTGCGACAGC GTGTCTGCTC

ACTGCCAGGA TGCAAGCTAC CACAAGAATC TCATCGCTGA TGCTATATAC TACCGAAAGA

ATGCCCATCG CCACAACACT GACACCACTC AGGCGACGGT AATGGCCATT GCCCTCCCTC

GAGGTCCTGC CCATGGTGCG AAAGCGAGGT ACAAGATCGA TAACAGCCCC ACTAAAGCTC

CGGCTGGGGT GTGCTGTTAG CTTCGCTGAG CAGAGGGTGA CACCTTTGTT CGACATCCGA

CTTACCGATT GATCAGCTGG TGGCCAGTTC AGTGCCTTCT TGCCATCTGG GTGCTTGATT

TGGGCGAGGT CGGGAATGGC GATGGTGGTC GCGACGAAGC AGGCGAGCAG AGTTGTCAAG

AGTTGAAACT TCATGGTTGC GACGGTGTTT CTTGATTATG GATTTCGAAG TGCGACAAGG

CGGACTGTGA GGAATATGTT GCAAGTGTTT GCTAGTATTT GCAGACGACC AAAGTGGTTT

GAAACGAATG CTGAGTGGGT GCTCGGAAGA GGTGAGGTCA AGTTCGCCCG ACGAAGCATG

GAATCCCGAA TCCCGATGGG AACTCAAAGC GTCCGAATCA GAGGCTGCAG TAGTCGGACC

TGCACAACGA CGCTGCCACT TCCGACCTTT ATTGTCTGGC AACATCATTG TCACTGTCTC

GCAAATGGCT GTGGTAGTGT TGTATCGCAG GACGCGCGCA CGGCTAGTCG CTGTCCGGAT

CCAGCGTCTT GCCCACGACC GCTGTCCTTC CACGGACCTG AGCAAAGCCA CGACTCTTTA

TCACTGCTAC CGTCTCGCTT GATACATTGA GACTCGTACA TCACACTCAC TGCTTCCTTC

GTACCCCTTA CCGGCGCCAC AACAGACACC ACCTCACCTC ACACTACTAC ACACACCACC

5' flanking region

→ *dotA* translation start point.

TCATCTCCCA TAATGTCCGT CGACAACTTC CGCCTCGACG GCAAGGTCGC CCTCGTTACT

GGATCTGGCC GTGGTATCGG TGCTGCCATC GCCATCGAGC TCGGCAAGCG TGGCGCAAAC

GTCGTCGTCA ACTACTCGCG AGCCGTCGCA GAGGCCAACA AAGTCGTCGA GACCATCATC

GCCAACGGCA CCAAGGCCAT CGCCATCAAG GCCGATGTCG GTGAGATTGA CCAGGTCGCG

AAGATGATGG ACCAGGCTGT TGAGCACTTT GGTCAACTCG ATATCGTCTC TTCGAACGCT

GGGCTTGTTT CTTTTGGACA TTTGAAGGAT GTCACTGGTG ATGTACGTTG TAAACATTTG

TCTTTCGTTT GAATGGATGA GAGCACAGAT GGCTGATTTA TCAAACAGGA ATTCGACCGC

GTCTTCCGTG TCAACACCCG AGGTCAATTC TTTGTCGCTC GCGAGGCGTA CCGCCACCTC

AGCGTCGGCG GCCGCATCAT TCTCACATCC TCAAACACCG CCTCCATCAA GGGTGTTCCC

AAGCACGCCA TCTACTCTGG CTCCAAGGGC GCCATCGACA CCTTTGTCCG CTGCATGGTA

TGTCCCCAAA TCACCTCGAA ACCCTCGCAA ACCACCCCAA GCTAATCATA CCTAGGCCAT

CGACGCCGGT GACAAGAAAA TCACCGTCAA CGCCGTCGCT CCCGGCGCCA TTAAGACTGA

CATGTACGCG GCCGTCGCGC GCGAGTACAT CCCCGGTGGC GACAAGTTCA CTGATGAGCA

AGTCGACGAG TGTGCTGCTT GGCTATCGCC GCTGGAGCGT GTAGGTCTGC CGGCGGACAT

TGGTCGTGTG GTCTGCTTCC TTGCATCGGA TGCAGCGGAG TGGGTCAGTG GGAAGATTCT

TGGTATTGAC GGTGGTGCTT TCCGATAGGT GTGAAGTCTT TGAACTGGGA ACTGGAGAAG

3' flanking region

dotA translation stop codon *

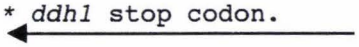
AAGTAAGTTT CTTTTGCTGT TGGCATCTAG CGGACACTAG ATTCCATGAT AGAACGCACC

GGGTATCTAT CCTCCATGAG TGGAGTGTTA TGGTCGATGT GATGTCTAGT GACATATCTC

ATTTACTACC GATGCAAACA AGCTATTACT CCATGACGGC GAGGATCGTC TTCCAGCATG

CCCCATATGA ACCTCATCTC *TGCACATACC*

* *ddh1* stop codon.



APPENDIX 4: Data and calculations for dothistromin quantification.

Table A4.1 Values and calculations for construction of standard curve.

[dothistromin] ng/ml	Log [Dothistromin]	Absorbance at 492nm			Average % Inhibition.
		Sample 1	Sample 2	Average (B)	
0.75	-0.125	0.959	0.918	0.939	-0.724
1.5	0.176	0.904	0.895	0.900	3.461
3.75	0.574	0.872	0.870	0.871	6.520
7.5	0.875	0.834	0.784	0.809	13.174
15.5	1.190	0.674	0.750	0.712	23.585
31.2	1.494	0.535	0.632	0.583	37.376
62.5	1.796	0.459	0.413	0.436	53.206
125	2.097	0.298	0.302	0.300	67.803
250	2.398	0.177	0.193	0.185	80.145
500	2.699	0.099	0.104	0.102	89.107
1000	3	0.065	0.067	0.066	92.917

% Inhibition = $(B_o - B)/B_o$. Where B_o= 0.93175 and is the mean absorbance of standards with 0 ng/ml dothistromin (n=8). These values were plotted on figure 4. and used to calculate dothistromin concentration in unknown samples.

APPENDIX 5: Statistical analysis to compare dothistromin production of strain 8A1 and NZE5.

t test to compare two means.

Null Hypothesis: H_o: $\mu_1 - \mu_2 \neq 0$

Test statistic: $$t = \frac{\bar{x}_1 - \bar{x}_2 - (\mu_1 - \mu_2)}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

The appropriate df for the two-sample *t* test is

$$df = \frac{(V_1 + V_2)^2}{\frac{V_1^2}{n_1 - 1} + \frac{V_2^2}{n_2 - 1}} \qquad \text{Where } V_1 = \frac{s_1^2}{n_1} \text{ and } V_2 = \frac{s_2^2}{n_2}$$

1 as subscript denotes colony 8A1 amd 2 as subscript denotes colony NZE5.
Results in Table 3.4.

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