Paxilline negative mutants of Penicillium paxilli generated by heterologous and homologous plasmid integration

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Carolyn Anne Young

1998

This Thesis is dedicated to

My Father

Tui Dene Young

1935 - 1992

Dad I wish you could be here today.
I know how proud you would be.
I miss you heaps and
Love you forever.

Carolyn

Abstract

Using a monoclonal antibody-based ELISA, 600 pAN7-1 plasmid-tagged mutants of *Penicillium paxilli* were screened for paxilline accumulation and one paxilline negative mutant, YI-20, was identified (Itoh, unpublished data). A molecular analysis of this mutant showed that pAN7-1 was inserted at a single site but was present as 4-6 copies arranged in a head-to tail tandem repeat. Rescue of flanking sequences and analysis of the corresponding genomic region revealed that YI-20 has an extensive deletion at the site of pAN7-1 integration. Probing of a CHEF gel with the same sequences showed that associated with the deletion is a rearrangement of chromosome Va. Targeted gene disruption of wild-type sequences adjacent to the site where pAN7-1 inserted, resulted in the generation of two additional paxilline-negative mutants; both were single crossovers with deletions extending outside the region mapped. Neither of these new mutants had a rearrangement of chromosome Va, suggesting that deletion of genes on this chromosome is responsible for the paxilline-negative phenotype.

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Introduction

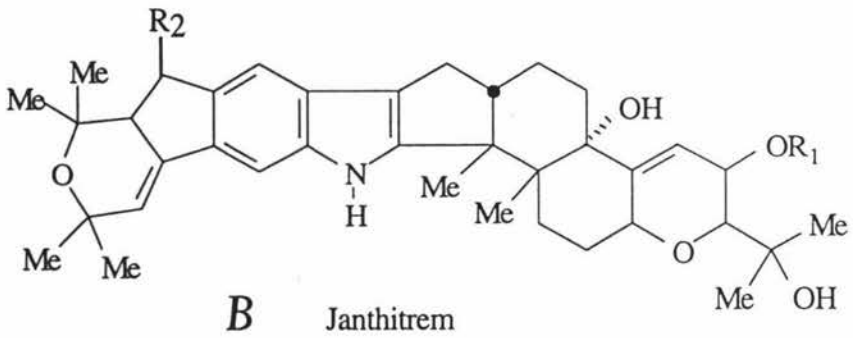
1.1 *Penicillium paxilli*

Members of the genus *Penicillium* are widespread throughout nature and most well known for their useful metabolite, the antibiotic penicillin. More recently, additional secondary metabolites have been isolated from *Penicillium*. Such metabolites have no known role in the organism's living processes (e.g. structure, replication, differentiation, communication and/or homeostasis) but are synthesized from either intermediates or end products of primary metabolism (Peberdy, 1987). A large group of these secondary metabolites, found not only in the *Penicillium spp.* but also in other filamentous fungi such as *Aspergillus spp.* (Gallagher and Wilson, 1978; Seya *et al.*, 1986; Nozawa *et al.*, 1987), *Claviceps spp.* (Cole *et al.*, 1977; Dorner *et al.*, 1984) and *Neotyphodium spp.* (Gallagher *et al.*, 1984) are known as tremorgenic mycotoxins. These toxins are fungal metabolites that affect the central nervous system function in vertebrates. An example of such a tremorgen is the indole-diterpenoid, paxilline, (Fig. 1.1) produced by *Penicillium paxilli*.

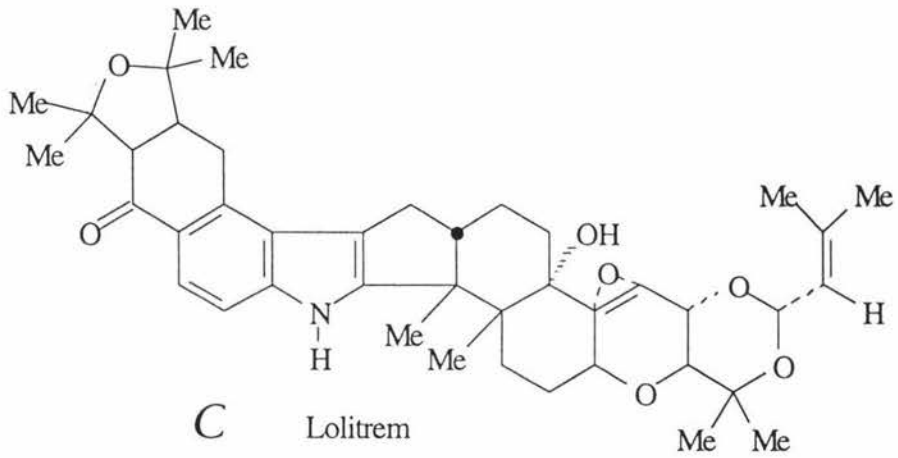
Penicillium paxilli, an asexual saprophytic fungi, was first isolated from insect-damaged pecans in the state of Georgia, USA (Cole *et al.*, 1974). This fungus grows rapidly, sporulates readily and produces large amounts of paxilline in submerged culture. Production of paxilline by *P. paxilli* was found to cause tremors when fed to both cockerels and mice (Cole *et al.*, 1974). Also reported, is the isolation of *P. paxilli* from the faeces of cattle in Victoria, Australia and soil from pastures in South Australia (Cockrum *et al.*, 1979). However, these two isolates did not produce the characteristic paxilline but substantial quantities of the tremorgenic toxin verruculogen, a prenylated diketopiperazine (Cockrum *et al.*, 1979). Although structurally different from paxilline, verruculogen contains both indole and isoprene moieties.

UV and IR spectra show that paxilline contains indole (Cole *et al.*, 1974) and its structure was determined by X-ray diffraction analysis. Data from labelling studies (Acklin *et al.*, 1977; de Jesus *et al.*, 1983; Laws and Mantle, 1989) show that both radiolabelled tryptophan and mevalonic acid are primary precursors of paxilline, where the incorporation of four mevalonate molecules indicates an involvement in isoprenoid biosynthesis (Fig. 1.2).

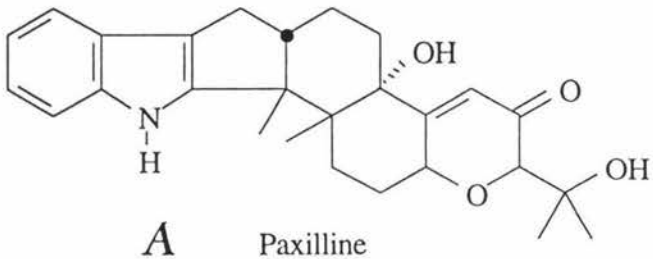
Secondary metabolites that contain tryptophan derivatives usually retain the aliphatic side chain. However, this tremorgenic mycotoxin has lost both the α- and β-carbons (Peberdy, 1987). Other such mycotoxins, which contain a single nitrogen atom as part of an indole nucleus and also have similarities with much of the indole-diterpene structure,



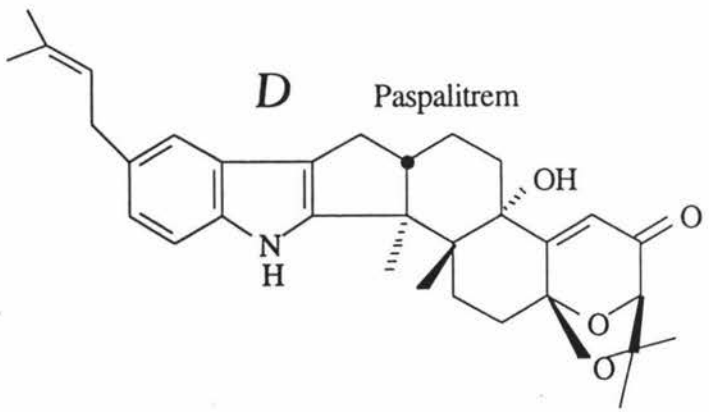
B Janthitrem



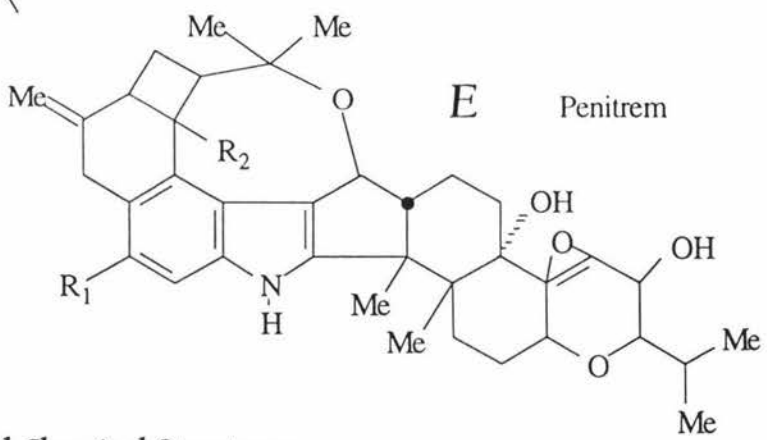
C Lolitrem



A Paxilline



D Paspalitrem



E Penitrem

Fig. 1.1 *Indole-diterpenoid Chemical Structures*

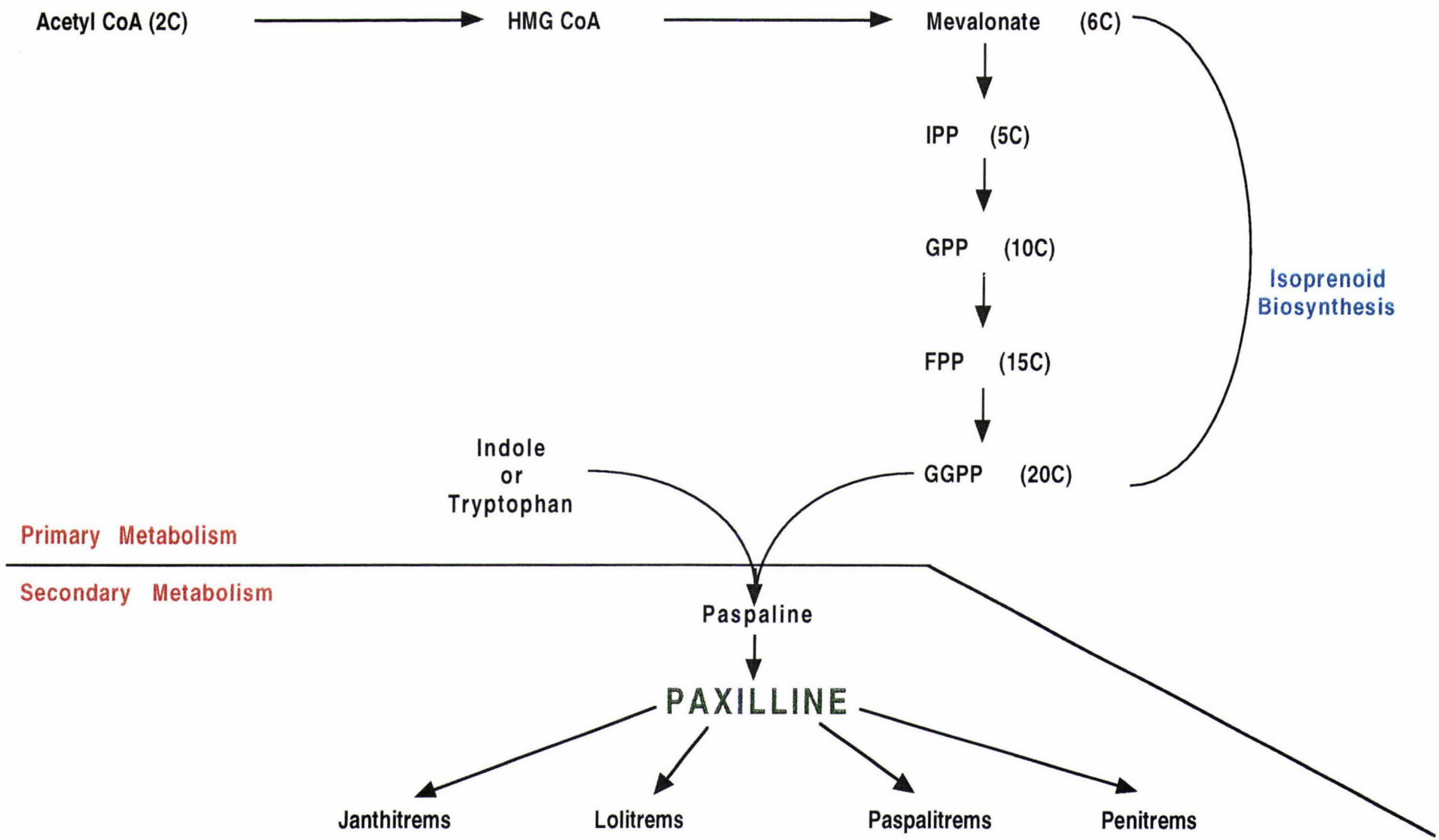


Fig. 1.2 *The Proposed Indole-Diterpenoid Pathway*

A schematic diagram showing the boundaries of primary and secondary metabolism within the indole-diterpenoid biosynthetic pathway.

have been grouped into four structural classes (Fig. 1.1 and 1.2): (i) the janthitrems (Gallagher *et al.*, 1980), (ii) the lolitrems (Gallagher *et al.*, 1984), (iii) the paspalitrems (Cole *et al.*, 1977; Dorner *et al.*, 1984) and (iv) the penitrems (de Jesus *et al.*, 1983). Although arising from a diverse range of fungal species, all of these compounds appear to share a common structural backbone of paxilline (Fig 1.1) and are known to be tremorgenic. Paxilline has been readily found in a range of fungi known to produce these indole-diterpenoids (Seya *et al.*, 1986; Weedon and Mantle, 1987), and labelling studies using [^{14}C]paxilline have shown that it is incorporated into the penitrems (Mantle and Penn, 1989), supporting the theory that paxilline is a key intermediate for these indole-diterpenoids.

Munday-Finch *et al.* (1996) have proposed a metabolic grid for paxilline production where the initial reaction must be prenylation of indole with geranylgeranyl diphosphate, a 20 membered carbon isoprenoid. Beyond this point, by looking at the individual structural changes between each of the intermediates it maybe possible to predict the type of enzymes that would be associated with this pathway.

1.2 *Paxilline Biosynthesis*

1.2.1 *Primary Metabolism - The Indole Moiety*

It has long been thought that the indole group of the indole-diterpenoids is incorporated from tryptophan that has lost the aliphatic side chain possibly by tryptophanase activity. This theory has arisen from the fact that tryptophan synthase catalyses the reaction of indole-3-glycerol phosphate to tryptophan via an indole intermediate that is not released (Stryer, 1995). Tryptophan synthase is a bifunctional enzyme with two different domains, an α-subunit (TSA) and a β-subunit (TSB). TSA catalyses the first reaction of indole-3-glycerol phosphate to indole. However, the indole is never released but passes down a 'tunnel' to the β-subunit. It is in TSB that indole is converted to tryptophan and released. The role of tryptophan synthase means that any available indole is sequestered and, therefore, tryptophan could be the only precursor for indole-diterpenoid biosynthesis. However, Frey *et al.* (1997) have recently shown that in addition to the tryptophan synthase α- and β-subunits there is evidence for an independently acting TSA subunit involved in the biosynthesis of two cyclic hydroxamic acids that form part of the defence against insects and microbial pathogens of maize. As labelling studies to date (Acklin *et*

al., 1977; de Jesus *et al.*, 1983; Laws and Mantle, 1989) have not addressed the issue of direct incorporation of indole, it is possible that a TSA-like enzyme is responsible for the indole supply instead of tryptophanase. If such a TSA enzyme was involved with paxilline biosynthesis, it would have to be dedicated to this pathway, therefore, there would be a need for two TSA-like subunits within the genome. One would be associated with primary metabolism and the TSB subunit producing tryptophan while the other, remaining independent and used for secondary metabolism.

1.2.2 *Primary Metabolism - The Diterpenoid Moiety*

Isoprenoids are a structurally diverse group of compounds (Chappell, 1995), which can be divided into primary (e.g. sterols, gibberellic acid, carotenoids and those involved in protein prenylation) and secondary metabolites (e.g. monoterpenes, sesquiterpenes and diterpenes). The isoprenoid pathway involves the sequential condensation of three acetyl-CoA units to generate 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), which is converted to the six carbon mevalonate in an irreversible reaction catalysed by HMG-CoA reductase (Fig. 1.2) Isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP), are generated by phosphorylation and decarboxylation of mevalonate. It is IPP and DMAPP that are the five-carbon building blocks for all other isoprenoids. Condensation of IPP and DMAPP in a head-to-tail manner generates geranyl diphosphate (GPP); addition of another IPP molecule generates farnesyl diphosphate (FPP) and addition of a third IPP molecule generates geranylgeranyl diphosphate (GGPP). The polymerisation reactions are catalysed by a series of enzymes known as prenyltransferases that are associated with primary metabolism (Fig. 1.2). The group of enzymes responsible for cyclisation of GPP, FPP and GGPP, the terpene, sesqui- and diterpene cyclases, utilise a similar reaction mechanism to the prenyltransferases.

Recent studies in *Neurospora crassa* (Barbato *et al.*, 1996) using RIP (Repeat-Induced Point Mutation) suggests that a complete knockout of the geranylgeranyl diphosphate synthase gene (*al-3*) would be lethal. It appeared that RIP would not introduce more than six point mutations and these mutations were leaky, leaving a partially functional enzyme. Disruption of the geranylgeranyl diphosphate synthase gene (*BST1*) in *Saccharomyces cerevisiae* (Yiang *et al.*, 1995) showed that *BST1* was not essential for the vegetative growth of the yeast cells but, in its absence, growth is impaired at temperatures of 25°C and below. These results suggest that a knockout of geranylgeranyl diphosphate synthase would result not only in the loss of indole-diterpenoid biosynthesis but also would have a large effect on the fungal growth.

1.2.3 *Secondary Metabolism - Paxilline Production*

The putative pathway for paxilline biosynthesis (Munday-Finch *et al.*, 1996) has been deduced by the presence of likely chemical intermediates and logical progression of the chemical structures. It is thought that paxilline biosynthesis must begin with the prenylation of indole or tryptophan with GGPP, as well as cyclisation of the 20 carbon isoprenoid unit by a diterpene cyclase or synthase resulting in paspaline. Paspaline is oxidised to paspaline B which undergoes oxidative decarboxylation followed by allylic oxidation to give paxilline. Therefore, based on the need for progressive chemical oxygenation of putative intermediates, one would expect the involvement of oxygenases such as cytochrome P450 monooxygenases. Other expected enzymes include hydrolases and decarboxylases. However, until the pathway has been elucidated biochemically, proposed steps would be only speculative.

Due to the similar structures of paxilline and the other indole-diterpenoid compounds (Fig. 1.1) determination of the paxilline biosynthetic pathway should allow easy isolation of the genes responsible for the other pathways, such as the lolitrems, paspalitrems, penitrems and janthitrems.

1.3 *Gene Clusters*

Fungal gene clusters have been broadly defined as the close linkage of two or more genes that participate in a common metabolic or developmental pathway. Clusters commonly found are those of dispensable pathways such as nutrient utilization and the production of secondary metabolites (Keller and Hohn, 1997). These pathways are not required for the normal growth of the organism but are thought to be present for selectional advantages, in particular, as a response to nutrient deprivation or competing organisms. Gene clusters have only recently been found in filamentous fungi. Since the 1980s there has been genetic evidence, using pathway mutants, that established the likely clustering of some genes responsible for aflatoxin biosynthesis (Bennett and Papa, 1988). However, the first molecular evidence of a fungal gene cluster did not arise until 1989, when Hull *et al.* (1989) found the L-proline catabolic pathway of *A. nidulans*.

As many prokaryotic metabolic pathways are clustered and due to similarities seen between some of the fungal and prokaryotic pathways, it is proposed that some fungal-pathway gene clustering may have evolved from horizontal gene transfer from prokaryotes. A good example of this is the isopenicillin N synthase gene from *Streptomyces lipmanii* and *Aspergillus nidulans,* where similarities also include the lack of introns and high GC content (Weigel *et al.*, 1988).

1.3.1 *Dispensable Catabolic Pathways*

To date, four dispensable catabolic pathways (quinate, ethanol, proline and nitrate utilisation) are found to be clustered in the filamentous fungus *A. nidulans* (reviewed by Keller and Hohn, 1997). These clusters are in the size range of 12 - 18 kb and appear to contain genes encoding biosynthetic enzymes, transcription factors and transporters. Although some of these pathways are clustered in other fungi, this is not always the case. For example, proline utilisation in *S. cerevisiae* is not clustered, while protein function is, however, still conserved.

1.3.2 *Secondary Metabolite - Penicillin*

Since the Second World War there has been a large focus on the production of the β-lactam derivatives, the penicillins and cephalosporins. This would make the penicillin biosynthetic pathway the most well studied of all the secondary metabolites produced by filamentous fungi. Although chemists have tried to establish commercial chemical synthesis of the penicillins, the highly sensitive β-lactam ring does not withstand this, leaving fermentation as the most efficient way of isolating large quantities of penicillin. It was in the 1960s that isolation of linear δ-(α-aminoadipyl)-cysteinyl valine (ACV) from *P. chrysogenum* gave the first evidence that penicillin was a tripeptide made of cysteine, valine and α-aminoadipic acid. In the 1980s the penicillin genes were isolated, with the first evidence of a gene cluster adduced in 1989 by Barredo *et al.*, (1989).

The penicillin gene cluster of *Penicillium chrysogenum* is approximately 20 kb, containing three genes that encode the biosynthetic enzymes, ACV synthetase, isopenicillin N synthetase and acyltransferase. These genes are also known to be clustered in *A. nidulans* and in the cephalosporin-producing *Acremonium chrysogenum.*

1.3.3 *Secondary Metabolite - Trichothecenes*

Fusarium, a fungus known to cause dry rot in potato tubers, produces a family of toxic sesquiterpenoids, the trichothecenes. These mycotoxins accumulate in agricultural products, are potent inhibitors of eukaryotic protein synthesis and are thought to be involved with plant diseases. Trichothecenes are formed from the primary metabolite, farnesyl diphosphate (FPP) by cyclisation catalysed by trichodiene synthase followed by subsequent modification of trichodiene through a series of oxygenation steps. Isolation of trichodiene synthase showed that it is the first committed step in trichothecene biosynthesis (Beremand, 1987; Hohn and Beremand, 1989). Evidence of a gene cluster was shown by Hohn *et al.* (1993) with the isolation of two overlapping cosmid clones that complemented mutants which blocked the trichothecene pathway. To date, the gene cluster from *Fusarium sporotrichioides* is thought to be ~25 kb with nine genes. Of the six biosynthetic enzymes, two share sequence identity with cytochrome P450 monooxygenases, three share sequence identity with acetyltransferases and the other, based on enzyme activity, is trichodiene synthase. The three remaining genes include a transcription factor that is required for pathway expression (Proctor *et al.*, 1995), an apparent transport protein, and one gene with no known function (Keller and Hohn, 1997).

1.3.4 *Secondary Metabolite - Aflatoxin/Sterigmatocystin*

The polyketide mycotoxins, aflatoxin and sterigmatocystin, are potent carcinogens produced by the fungi *Aspergillus flavus, A. parasiticus* (aflatoxin) and *A. nidulans* (sterigmatocystin). These fungi infect corn, cotton and peanuts and are a major threat to the health of humans. Evidence for a gene cluster in *A. parasiticus* was based on the isolation of the closely linked aflatoxin genes, *ver-1* and *nor-1* (Skory *et al.*, 1992), while physical mapping of the *A. flavus apa-2,* an aflatoxin regulatory gene, placed it approximately 8 kb away from *ver-1* (Chang *et al.*, 1993). Keller *et al.* (1994) found evidence that the sterigmatocystin pathway is clustered, when they isolated the *ver*A gene from *A. nidulans*. Two other open reading frames were found alongside the *ver*A gene, one was predicted by sequence identity to encode a P450 monooxygenase (Keller *et al.*, 1994). As with the trichothecene pathway, such genes are expected to be involved with sterigmatocystin and aflatoxin synthesis. The complete sterigmatocystin biosynthetic cluster of *A. nidulans* has been published (Brown *et al.*, 1996) with similarities between the two gene clusters of aflatoxin and sterigmatocystin reviewed by Keller and Hohn

(1997). Although the order of the genes is not identical, the two clusters possess similar gene function and regulatory control. The 60 kb sterigmatocystin cluster contains 25 genes; 16 encoding biosynthetic enzymes, one regulatory gene and eight with unknown functions. Of the 16 biosynthetic enzymes, five show similarity to P450 monooxygenases, four are dehydrogenases and the remaining enzymes include an esterase, an *O*-methyltransferase, a reductase and an oxidase. The boundary of the gene cluster was determined by Northern analysis as fragments that hybridised under both sterigmatocystin-inducing and non-sterigmatocystin-inducing conditions (Brown *et al.*, 1996). At present it is the aim of these groups to knockout each of the genes individually to determine their function.

1.3.5 Secondary Metabolite - Melanin

Melanins are high-molecular-weight, coloured pigments produced by numerous fungi. They give the fungus protection from desiccation, UV irradiation and extreme temperatures as well as cell wall rigidity. They are also thought to be associated with the ability of certain fungal plant pathogens to penetrate host plant cells. Melanins are formed by the oxidative polymerisation of phenolic compounds with the precursors being either polyketide products or tyrosine. A three-gene cluster containing a polyketide synthase, scytalone dehydratase and 1,3,8-trihydroxynaphthalene synthase was identified in the non-pathogenic fungus *Alternaria alternata* (Kimura and Tsuge, 1993). However, with the pathogenic fungi *Colletotrichum lagenarium* and *Magnaporthe grisea,* for example, these genes are not clustered.

1.3.6 Secondary Metabolite - Paxilline

Paxilline, is a known secondary metabolite from *P. paxilli*, that is not required for normal growth and development of this fungus. To date, there is no genetic evidence available, to show that the paxilline biosynthetic genes are clustered. However, as many fungal secondary metabolites are clustered (Section 1.3.1 - 1.3.5) it is also thought the paxilline biosynthetic genes maybe.

1.4 *Approaches to Cloning Secondary Metabolite Genes*

1.4.1 *Mutagenesis*

UV and chemical mutagenesis have been used for a number of years to isolate a large number of mutants. The advantage of such a system is that a large quantity of random mutants can be quickly isolated with no knowledge of the genes involved. Although easy to perform, there are several drawbacks for using random mutagenesis: (i) there is the possibility of more than one mutation per genome; (ii) the mutations are not tagged in any way making it difficult to isolate the mutated gene; (iii) large numbers of mutants have to be screened to identify the phenotype of interest. However, once mutant has been isolated, several strategies can be used to identify the gene responsible. Beremand (1987) used UV mutagenesis to generate 1000 mutants of *Fusarium sporotrichioides*. Using a microtitre plate to grow the cultures, Beremand (1987) was able to rapidly screen these mutants by a monoclonal antibody to trichothecene. The three mutants identified were characterised chemically for their role in trichothecene biosynthesis. Complementation with a cosmid library of *F. sporotrichioides* genomic DNA containing a hygromycin selectable marker was used to rescue two trichothecene-deficient mutants (Hohn *et al.*, 1993). The cosmids were characterised further to determine the sequences responsible for complementation of the different mutations.

An alternative approach is that of heterologous plasmid tagging by integrative transformation of a plasmid that has no homology with the fungal genome. The plasmid, such as pAN7-1 (Punt *et al.*, 1987), contains a fungal selectable marker (e.g. a gene for hygromycin resistance) and is thought to integrate into the genome of the fungal protoplasts by illegitimate recombination at the site of chromosome nicks or breaks (Fig. 3A). More recently, plasmid tagging has been modified by the addition of a restriction enzyme (Restriction Enzyme Mediated Integration - REMI) during the transformation process (Schiestl and Petes, 1991). A plasmid carrying a fungal selectable marker is linearised with a suitable restriction enzyme and used to transform fungal protoplasts in the presence of the restriction enzyme. Integration of the plasmid occurs at the restriction enzyme target site presumably due to cleavage with the enzyme (Fig. 1.3B). This plasmid tagging method has proven very successful with a number of fungi and is found to increase the transformation frequency and favour the integration of the plasmid as a single copy. REMI was shown to increase the *Cochliobolus heterostrophus* transformation frequency from 6 up to as many as 107 transformants per 30 μg of linear DNA per 10^6

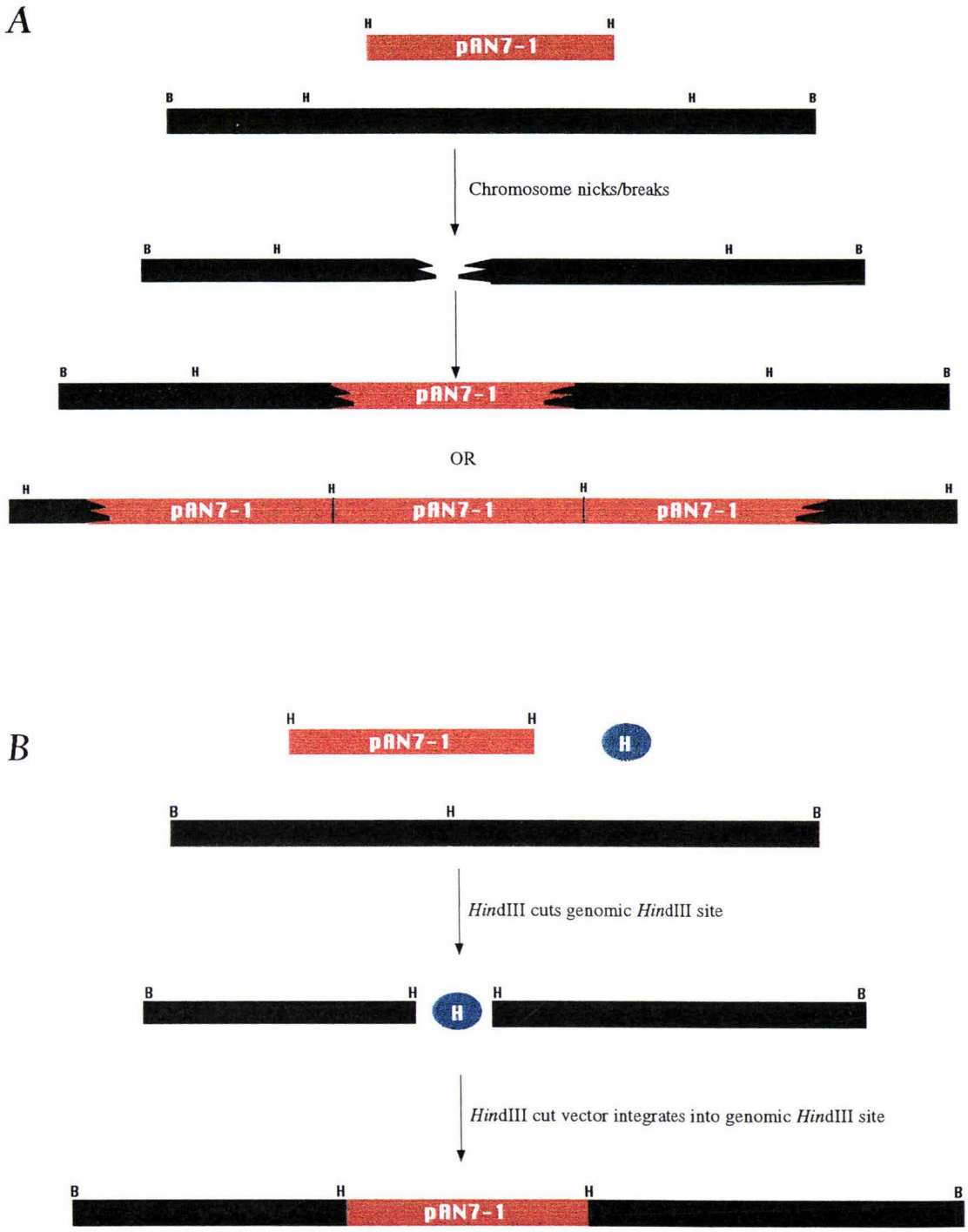


***Fig. 1.3** Plasmid Tagging*

A Illegitimate recombination

B Restriction Enzyme Mediated Integration (REMI)

The integrating plasmid (pAN7-1) is red. The restriction enzyme is blue. Genomic DNA is black. Abbreviations: B, *Bam*HI. H, *Hin*dIII.

protoplasts when a restriction enzyme was included (Lu *et al.*, 1994). Of the 1310 transformants isolated, two failed to produce detectable T-toxin and virulence to maize was lost. Characterisation of these mutants showed the Tox$^-$ mutation of the two transformants mapped to the expected *Tox1* locus (Lu *et al.*, 1994). DNA recovered from the insertion site of one mutant encodes a 7.6 kb open reading frame that identified a multifunctional polyketide synthase (Yang *et al.*, 1996). Analysis of 1000 REMI insertion mutants of the maize pathogenic fungus *Ustilago maydis* demonstrated that 1-2% of these mutants were unable to induce symptoms when tested *in planta*. Characterisation of two of these mutants showed that the phenotype is linked to the insertion event (Bölker *et al.*, 1995).

With all mutagenesis techniques there is a requirement to screen large numbers of mutants to find the phenotype of interest. Therefore, an observable phenotype is very useful, for example, spore pigmentation (Itoh and Scott, 1994) or an ability to produce carotenoids such as those that give *N. crassa* its orange colour (Barbato *et al.*, 1996). However, for most mutants an observable phenotype is not available. More time-consuming screens involve *in planta* assays for pathogenicity (Bölker *et al.*, 1995) or extraction of the compound of interest for analysis by TLC (Hodges *et al.*, 1994). Monoclonal-based ELISA are useful for quick screening of culture filtrates for compounds of interest (Beremand, 1987). Such techniques have a quick assay system. However, the antibody needs to be reasonably specific for the compound being observed.

The advantage of plasmid tagging over the other mutagenesis techniques is the ability to rescue the tagged gene via plasmid rescue. Of course, there is still the labour intensive screen for transformants with the appropriate phenotype, as is also required for UV mutagenesis.

1.4.2 *Enzyme Isolation*

When an enzyme has a known biochemical activity that can be assayed it is possible to isolate the protein. A number of secondary metabolite genes have been found as a result of protein purification. Tricodiene synthase, the enzyme that catalyses the isomerisation and cyclisation of FPP to tricodiene, was isolated from *Fusarium sporotrichioides*. Antibodies to this enzyme were made and used to screen a λgt11 expression library to *F. sporotrichioides*. The resulting cloned DNA was sequenced and compared to the known protein sequence from CNBr cleaved fragments, confirming that the gene was tricodiene synthase. An internal fragment from the cDNA was used to isolate a cosmid containing the complete gene sequence. Expression of this gene and biochemical analysis confirmed the

tricodiene synthase activity (Hohn and Beremand, 1989). Tsai *et al.* (1995) used a slightly different approach. The isolated enzyme, dimethylallyl tryptophan synthase (DMAT), was first purified from mycelia of *Claviceps purpurea* (Gebler and Poulter, 1992). This enzyme is responsible for the alkylation of L-tryptophan by dimethylallyl diphosphate and is the first pathway-specific step of ergot alkaloid biosynthesis. Partial sequence of CNBr fragments was used to design degenerate primers that hybridised to *C. purpurea* genomic DNA. PCR was used with these primers and the nucleotide sequence compared to the available DMAT peptide sequence. The complete DMAT gene was cloned and sequenced, and its function confirmed by expression in a yeast background.

For the above method to be useful, protein sequence data are required. These are not always available and enzyme purification can be a lengthy process even with a biochemical assay. With the sequence databases increasing rapidly in size everyday, it is often possible to obtain and compare known protein sequences. If there is sufficient identity between the proteins, degenerate primers can be designed and used for PCR. The drawback of this system is finding regions of the sequence that contain enough identity with amino acids that have low codon usage. Unfortunately many proteins have similar activity but sequence conservation can be limited to a catalytic domain or an active site.

1.4.3 *Heterologous probing*

Aspergillus species are known to produce the similar compounds aflatoxin and sterigmatocystin. As a number of genes have been isolated from the aflatoxin-producing fungi, *A. parasiticus* and *A. flavus*, it is possible to use these cloned fragments for the isolation of homologues in *A. nidulans*. The *ver-1* gene from *A. parasiticus* is responsible for the enzymatic conversion of versicolorin A to sterigmatocystin (Skory *et al.*, 1992). Heterologous hybridisation was used to isolate *ver*-1 homologue from *A. nidulans* (Keller *et al.*, 1994). The hybridising fragment was subcloned from a cosmid and sequenced.

This technique has also been used for the isolation of dothistromin genes. Dothistromin is a polyketide toxin produced in pine by the fungus *Dothistroma pini.* As the chemical structure of dothistromin is similar to aflatoxin, similar enzymes maybe responsible for its production. Heterologous probing with the *ver-1* gene from *A. parasiticus* was used to isolate the *D. pini* homologue from a genomic library (Gillman and Bradshaw, personal comm.). Sequence data show that the *D. pini ver* gene has 75% identity to the *A. parasiticus* gene (Monahan and Bradshaw, personal comm.).

1.4.4 *Differential screening*

In recent years, there has been a number of RNA-based techniques established for looking at differentially expressed genes. Different culture conditions (e.g. temperature, time and nutrient sources) can induce or repress gene expression, these conditions are then used for RNA isolation. The earliest of these techniques was subtractive hybridisation. Feng *et al.* (1992) used growth phase, carbon source and temperature to distinguish differential expression of genes related to aflatoxin biosynthesis. The three labeled cDNA probes were hybridised successively to the same membranes hosting the DNA library. When compared, 0.6% of the 20 000 colonies were found to differentially hybridise to the three probes. These clones were screened against Northern blots to further prove their expression pattern.

Another differential screening method is that of differential display (DD) (Liang and Pardee, 1992). This is a PCR-based technique that compares differences in mRNA content between two different states. Total RNA isolated from cultures grown under different physiological conditions is amplified by reverse transcriptase with poly-T tailed anchored primers. These primers anneal to the poly-A tail of the mRNA and are anchored at the 3' end by A, C or G nucleotides. The second round of PCR uses the anchored poly-T primer with a primer of arbitrary sequence. This reduces the pool of represented mRNA so the fragments can be separated on a denaturing gel. The bands from the different growth conditions are compared to find fragments that are differentially expressed. These bands are isolated, reamplified and used to screen Northern blots to confirm their expression pattern.

1.4.5 *Proteonome Analysis*

There have been great advancements with two-dimensional (2D) gel electrophoresis of proteins, and with NMR and mass spectrophotometry for obtaining a protein sequence from a small sample. This has resulted in a revival in proteonome analysis, where the proteins of two physiological states are compared by 2D gel electrophoresis. Any proteins that are differentially expressed can be sequenced by mass spectrophotometry directly from the gel. It is also possible to determine protein function by comparison of 2D gel libraries of known proteins.

1.5 *Genetic Criteria to Confirm Functionality of the Isolated Gene.*

Once a potential gene is isolated, there needs to be a way of confirming its actual physiological role. In sexual fungi, crosses can be used to determine whether the phenotype and marker co-segregate. However, this approach cannot be used with asexual fungi and, therefore, other methods are required. In many cases, complementation can be used when a gene has been knocked out. When replacing it with a functional gene, the wild-type phenotype should be restored. This is not always possible due to the lack of available selectable markers for filamentous fungi. When complementation is not possible, homologous recombination can be used to knockout the gene of interest in a wild-type strain, resulting in a recessive phenotype. This approach can be lengthy as the double crossover integration event required for a true knockout occurs at a low frequency and is locus dependent (Bird *et al.*, 1997).

1.6 *Cloning of the Paxilline Biosynthetic Genes in Penicillium paxilli by Plasmid Integration*

As the genes of many fungal secondary metabolite pathways are arranged in clusters, it would be of no surprise to find this the case for the paxilline biosynthetic genes. To isolate such genes from *P. paxilli* the approach initiated was that of heterologous plasmid integration using a monoclonal-based competitive ELISA (Garthwaite *et al.*, 1993) to screen for the loss of paxilline production. If the genes responsible for paxilline biosynthesis are found in a cluster, random plasmid integration into one of these genes, would knock out paxilline production. Therefore, isolation of sequences flanking the plasmid integration site and the surrounding locus would result in the cloning of the clustered genes.

Itoh *et al.* (1994) used pAN7-1 (Appendix A1.1) to isolate 600 tagged transformants of *P. paxilli*. A sub-group of these transformants was checked for stability, frequency and randomness of integration. Up to 78% of the transformants contained the plasmid at a single site, and 50% of these transformants were present as a tandem repeat. The integration of the plasmid into the genome did not appear to be site specific. In contrast, work with *Aspergillus nidulans* using heterologous plasmid integration showed site-selectivity (Diallinas and Scazzocchio, 1989; Tilburn *et al.*, 1990). Within the 600

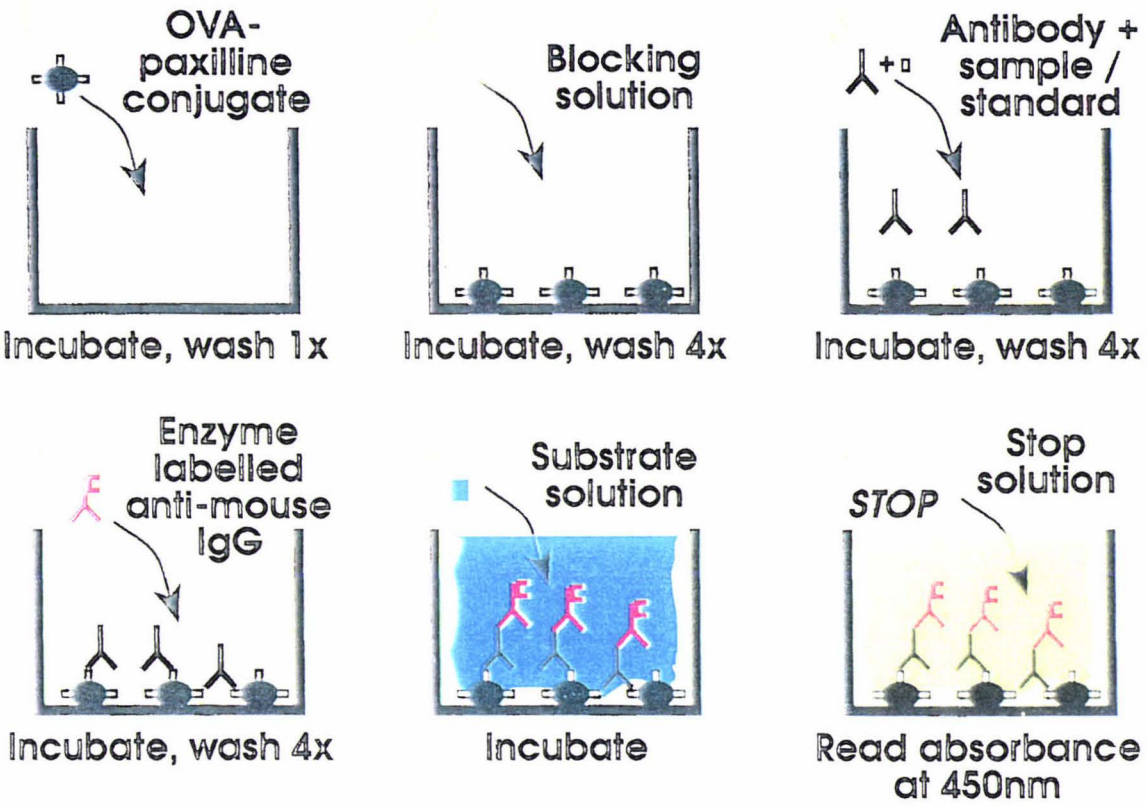


Fig. 1.4 *The Competitive ELISA for Paxilline*

Courtesy of Ian Garthwaite, Toxinology and Food Safety Research Group, Ruakura, Hamilton.

pAN7-1 tagged transformants, 0.83% had a characteristic brown spore phenotype. Isolation and characterisation of mutants in this spore pigmentation locus showed that the integration of pAN7-1 had caused extensive deletions through a common region of the genome (Itoh and Scott, 1994). All of the above mutants were also screened by the cELISA (Fig. 1.4) for their ability to produce paxilline in culture. The antibody used in this assay recognises both paxilline and an array of compounds structurally related to paxilline. One mutant, YI-20, had a paxilline negative phenotype as determined by cELISA and HPLC. Characterisation of this mutant by Southern analysis (Fig 1.5) showed that the integrating plasmid, pAN7-1, was present at a single site but in a head-to-tail tandem repeat. Hybridisation of genomic digests of YI-20 DNA with pAN7-1, shows bands present at 6.8 kb in the *Bam*HI (lane 2) and *Hin*dIII (lane 3) digests, indicative of a tandem repeat (Itoh, unpublished data).

1.7 *Aim*

The aim of this thesis is to characterise the Pax$^-$ mutant, YI-20, with respect to its integration of pAN7-1 and to establish whether plasmid integration was responsible for the phenotype observed. The genomic sequence surrounding the pAN7-1 integration will be isolated. These sequences will be used to make a replacement construct with the intent to generate additional paxilline-negative mutants via homologous recombination. The resulting mutants will be characterised for their ability to produce paxilline and for their integration events.

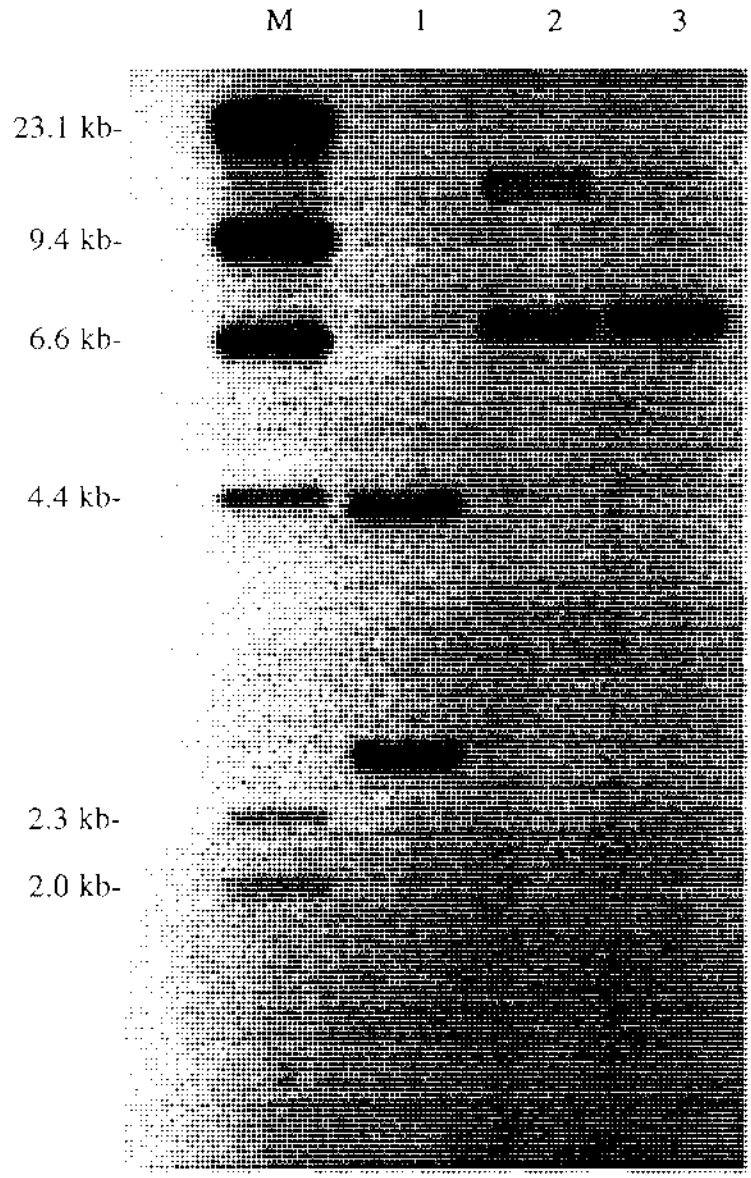


Fig. 1.5 *Southern analysis of the integration of pAN7-1 in YI-20*

Southern analysis of YI-20 genomic DNA digested with *Eco*RI (lane 1), *Bam*HI (lane 2), *Hin*dIII (lane 3), hybridised with pAN7-1. Lane M, λ *Hin*dIII.