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CLONING, CHARACTERISATION AND EVOLUTIONARY RELATIONSHIPS OF TWO *PYR*4 GENES FROM AN *ACREMONIUM* ENDOPHYTE OF PERENNIAL RYEGRASS

A thesis presented in partial fulfilment of
the requirements for the degree of
Doctor of Philosophy In Molecular Genetics
at Massey University, Palmerston North
New Zealand

Michael Anthony Collett

1994

ABSTRACT

A fragment of the *Claviceps purpurea pyr*4 gene, encoding the enzyme orotidine-5'-monophosphate decarboxylase (OMPdecarboxylase) was used to screen a genomic library to an isolate (designated Lp1) of an *Acremonium* sp. which grows as an endophyte in perennial ryegrass (*Lolium perenne*). Four positive clones, λMC11, λMC12, λMC14 and λMC20 were isolated. Three of these clones, λMC12, λMC14 and λMC20 were overlapping clones from the same locus, while λMC11 was from a different locus.

Fragments of these clones which hybridised with *C. purpurea pyr*4 were sequenced and found to have similarity with *pyr*4 from other fungi of the Pyrenomycetes and related Deuteromycetes, suggesting that Lp1 has evolved from a sexual Pyrenomycetes species. The *pyr*4 from λMC12, λMC14 and λMC20 was designated *pyr*4-1 and that from λMC11 was designated *pyr*4-2. The predicted ORFs of the two genes were highly conserved and the 5' non-coding nucleotide sequences were the least conserved regions.

RT-PCR and northern analysis of total RNA from Lp1 demonstrated that transcripts approximately 1.4 kb in length were produced from the two genes and present at similar levels. Genomic fragments containing *pyr*4-1 or *pyr*4-2 were transformed into a strain of *Aspergillus nidulans* which has a mutation in the *pyrG* gene (encoding OMPdecarboxylase). Both of the Lp1 *pyr*4 complemented a *pyrG* mutation in *Aspergillus nidulans*, confirming that both *pyr*4-1 and *pyr*4-2 encode functional OMPdecarboxylases.

Comparisons of *pyr*4 restriction fragment length polymorphisms (RFLPs) from Lp1 and isolates of *Epichloë typhina*, *E. festucae*, *A. lolii*, *A. uncinatum*, and three endophyte taxonomic groupings from *Festuca arundinacea*: FaTG-1 (=*A. coenophialum*), FaTG-2 and FaTG-3 suggested that *pyr*4-1 originated from *E. typhina*, the ryegrass choke pathogen, and *pyr*4-2 originated from *A. lolii,* another endophyte from perennial ryegrass. This suggested that Lp1 is an interspecific hybrid, between *E. typhina* and *A. lolii*. Comparisons of the variable 5' non-coding nucleotide sequences from *pyr*4 of Lp1 and other isolates demonstrated that *E. typhina*, and *A. lolii* or *E. festucae* were the most likely ancestors of the two *pyr*4 found in Lp1. The *A. lolii* and *E. festucae* sequences were very similar, suggesting they are closely related. *A. lolii* has most probably evolved from an *E. festucae*, and in the process lost the sexual cycle.

Analysis of single spore purified isolates of Lp1 revealed that Lp1 was a homokaryon for *pyr*4. A Southern blot of a CHEF gel of Lp1 and these single spored

isolates was hybridised to a *pyr*4 probe and demonstrated that *pyr*4-1 and *pyr*4-2 were present on either two chromosomes of similar size, or one chromosome.

The hybridisation that gave rise to Lp1 was concluded to have been a relatively recent event, given the similarity of *pyr*4-1 and *pyr*4-2 nucleotide sequences to those of their probable ancestors, and the fact that both genes are expressed and functional. Interspecific hybridisation is probably widespread in the asexual endophytes, and may be an important event in their evolution, and the evolution of other fungal species.

ACKNOWLEDGEMENTS

I would like to first offer my sincerest appreciation to my supervisor, Barry Scott, for excellent supervision and encouragement throughout all stages of this project. I would also like to thank my co-supervisors, Rosie Bradshaw for assistance and advice, particularly with RNA work, and Gary Latch for encouragement. Thanks also to the staff and students of the Department of Microbiology and Genetics, too numerous to mention here, for their assistance and encouragement. In addition I would also like to thank all members of the Molecular Genetics Unit, past and present, who have assisted me during my time here, thanks to all of you for making the Department and MGU an enjoyable (and interesting!) place to work. In particular I would like to thank Carolyn "bladder infection" Young and the other "Carolyn" (while the real one "recovered" from her infection), Dianne Watt for excellent technical assistance and advice on all things molecular and more besides during the course of this research. I would also like to thank Richard "rapid boil" Johnson, Yasuo "it just can not be helped" Itoh, Hale Nicholson, Austin "I'm not quite sure" Ganley, Sirinda "we're just friends" Yunchalard, Chris "PCR" Schardl, Celia Berkahn, Kim Gan, Robert Hickson, Grant Hotter and Cherie Stayner, for their much welcome and varied advice, encouragement and distractions offered over the course of this thesis. Thanks also to all tramping partners, for helping to keep me sane and alive (just). I would also like to offer my most grateful thanks to my family and friends for all their encouragement and help during this, often trying, three and a half years, I'm sorry if I've been notably inconspicuous during this time, but a PhD is like that some times. In particular I would like to thank Kate for all her love and support. It is not easy expressing my gratitude to all of you, but hopefully this goes some way towards that.

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Chapter 1.0 INTRODUCTION

1.1 THE BIOLOGICAL IMPORTANCE OF FUNGI

The fungi are an ecologically and biochemically diverse group of organisms (Rayner 1992). This made them outstandingly successful in many varied environments, and has resulted in them being an economically important component of many anthropogenic systems, having both beneficial and detrimental effects. This diversity has also led to confusion in the taxonomy of these organisms. In this thesis I will use the taxonomic system of Ainsworth *et al.* (1973). This system recognises two divisions of the subkingdom fungi. These are the Myxomycota ("lower fungi") and the Eumycota ("true fungi"). The division of most relevance to the work in this thesis, the Eumycota, is further divided into five subdivisions: the Mastigomycotina, which possess motile cells (zoospores); Deuteromycotina (Deuteromycetes), which lack a perfect (sexual) stage, and hence only reproduce asexually; Zygomycotina (Zygomycetes), whose perfect state spores are zygospores; Ascomycotina (Ascomycetes) whose perfect state spores are ascospores; and Basidomycotina (Basidomycetes) whose perfect state spores are basidiospores. The subdivisions of most relevance here are the Ascomycetes and Deuteromycetes. The Deuteromycetes are largely an artificial collection of fungi, which have lost their sexual stage. They are generally thought to have evolved from the Ascomycetes. The Ascomycetes are divided into six classes. Those of most relevance to this thesis are the classes Pyrenomycetes and Plectomycetes.

The beneficial activities of fungi are many, and include their use in the food and beverage industries. For example, *Saccharomyces cerevisiae* is widely used in brewing and baking, and *Agaricus bisporus*, the cultivated mushroom is harvested as an agricultural crop (Christensen 1965). Fungi are also important for the production of many pharmaceuticals and other biological compounds (Campbell and Carr 1979). A major role of the fungi is as primary decomposers in many ecosystems (Cromack and Caldwell 1992; Newell 1992), including agro-ecosystems. Thus they are an essential component of the carbon and nitrogen cycles, breaking down many complex biological macromolecules into simpler molecules, making them readily available for use by other, less metabolically complex, organisms such as plants and animals. In addition the fungi can form symbiotic associations with plants, animals and other micro-organisms (Smith 1979). These symbiotic associations can be classified as mutualistic, parasitic, or commensalistic, although symbiosis is interpreted by some workers to mean solely mutualistic. However, the classification of symbiotic associations is not always straight forward, and in some situations apparently mutualistic associations may become detrimental to either partner. It is probably more useful to describe the associations in

terms of a number of criteria such as durability (e.g. transient or permanent), type of interaction (e.g. extra or intracellular), degree of morphological interaction (production of specialised structures solely in the symbiosis), functional dependence (e.g. metabolic or reproductive dependence), specificity of symbionts for each other, dependence (facultative or obligate), nutrition (necrotrophic or biotrophic) and the value (mutualistic, neutral or antagonistic). The importance to man of many of these symbiotic associations has led to their widespread and detailed investigation (Alexander 1992; Allan and Allan 1992; Dickman 1992; Harley and Smith 1983; Read 1992, for example). In particular, associations of fungi and plants have been of enormous importance in agriculture. Fungi are major plant pathogens, with many diverse types of fungi attacking a wide range of plants (Agrios 1988). Investigations of these interactions have probably been the major focus of mycology to date. Probably equally important, but less noted, are the beneficial effects that fungi have on plants. Besides their vitally important role as primary decomposers in many ecosystems, fungi can be of great benefit to plants. Mycorrhizal symbiotic associations (between fungi and the root systems of plants) are widespread and well documented (Harley and Smith 1983). Another symbiotic association between plants and fungi that has received less attention, but is of considerable importance, is that of plants and endophytic fungi (i.e. those fungi growing entirely within the host plant, whether parasitic or not). This grouping is obviously extremely wide, including vesicular arbuscular mycorrhizas, biotrophic plant pathogens and a wide range of plant hosts. One particularly important group of endophytes are those generally found growing within the leaf sheaths of grasses, and which are often referred to simply as endophytes of grasses. In this thesis I will restrict myself to discussion solely of these endophytes, in particular the clavicipitaceous endophytic fungi that belong or are related to the tribe Balansieae.

1.2 ENDOPHYTES OF GRASSES

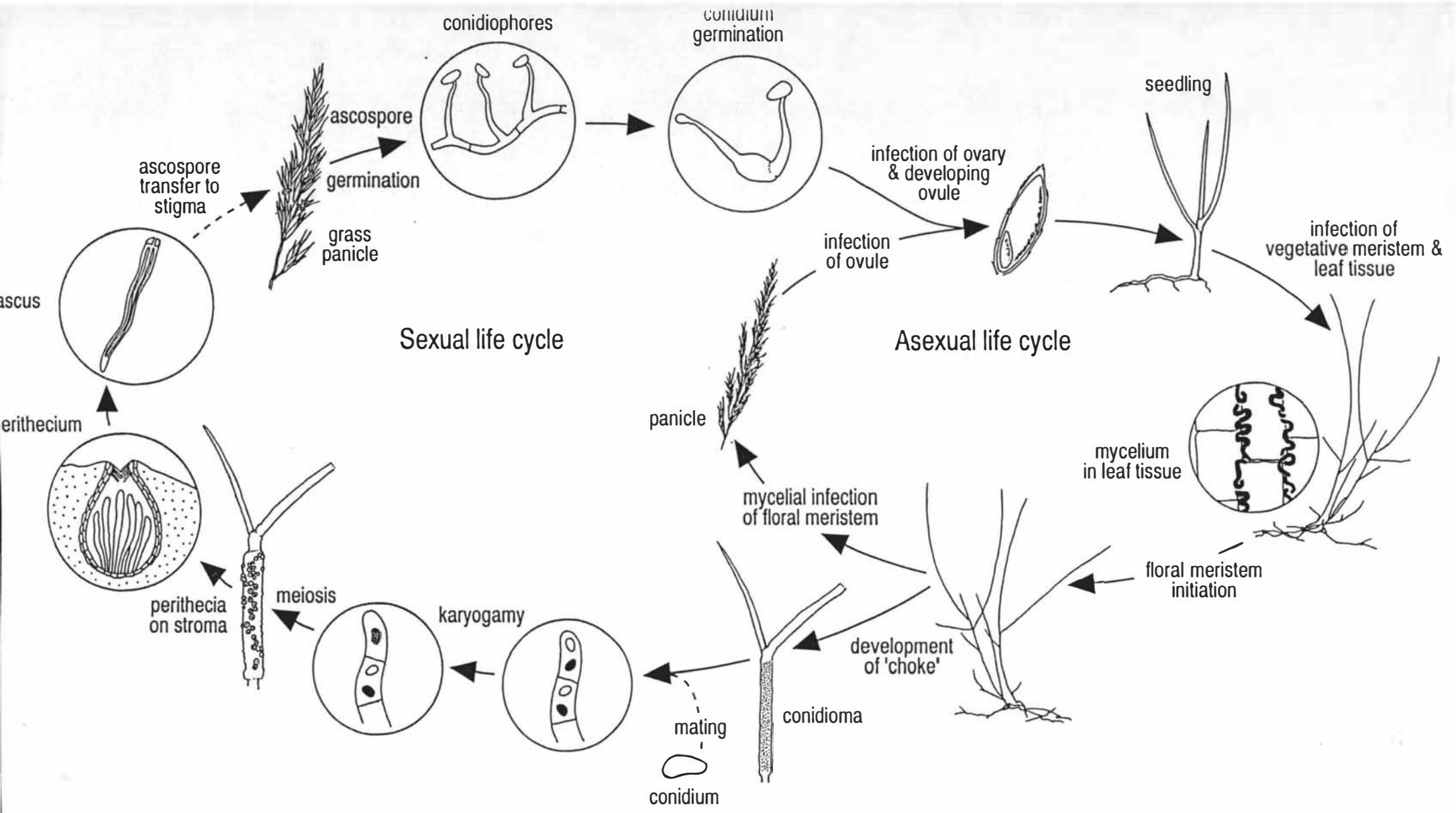
Clavicipitaceous endophytes are asexual filamentous fungi which form symbiotic associations with grasses of the subfamily Pooideae (White 1987). They are strictly mutualistic, conferring considerable benefits upon their grass hosts. These include protection from drought, plant pathogens, insect and mammalian herbivores as well as increased production of seeds and biomass (Clay 1990; Kimmons *et al.* 1990; Rice *et al.* 1990). The endophytes are found in the reproductive and vegetative aerial tissues of the host grasses. In natural associations they are not known to cause disease or produce external structures, apparently being perfectly adapted ecologically obligate biotrophs, which are transmitted within the seed of their hosts (Clay 1990; Latch *et al.* 1987, see Fig. 1 for life cycle). The most intensely documented of these symbioses are *Acremonium lolii* in perennial ryegrass (*Lolium perenne*) and *Acremonium coenophialum*

Fig. 1. Life cycle of an *Acremonium* endophyte and an *Epichloë festucae* growing symbiotically with tall fescue (*Festuca arundinacea*).

The sexual cycle of *Epichloë* spp. (represented here by *E. festucae*) is initiated by the emergence of fungal mycelia from the intercellular spaces and the development of a conidioma on the leaf surface. This conidioma 'chokes' the developing inflorescences. Following an insect mediated cross, from an *E. festucae* of the opposite mating type, sexual stromata (ascostromata) form (White and Bultman 1987). These bear orange-tan perithecia that, once mature, contain ascospores. These are thought to give rise to infective structures (see text). Following infection of the grass host *Epichloë* spp. infect the vegetative plant meristems, and from there are maintained in the plant by vegetative (asexual) growth in the intercellular spaces of the leaf sheaths. In natural associations there is no evidence for the presence of invasive fungal structures in plant cells. Following floral meristem development, the mycelia may either invade the ovaries and developing ovules, which results in eventual infection of the grass seed, or re-embark on the sexual cycle. Infected seeds give rise to infected plants, so the endophytes are maternally transmitted.

Acremonium endophytes (e.g. *A. coenophialum* or *A. lolii*) grow entirely asexually within the plant, and are incapable of choking the plant. If an *Epichloë* spp. should lose the ability to form the conidioma, it would become an asexual endophyte.

Courtesy of Liz Grant, Department of Ecology, Massey University.


conidiophores
conidium germination
ascospore
germination
ascospore transfer to stigma
grass panicle
infection of ovary & developing ovule
infection of ovule
seedling
infection of vegetative meristem & leaf tissue
ascus
Sexual life cycle
Asexual life cycle
panicle
mycelium in leaf tissue
erithecium
mycelial infection of floral meristem
floral meristem initiation
perithecia on stroma
meiosis
karyogamy
development of 'choke'
conidioma
mating
conidium

in tall fescue (*Festuca arundinacea*). These two grass species are major forage crops in grazing agro-ecosystems. Endophyte infected ryegrass and tall fescue is widespread in both New Zealand and the United States respectively. The importance of the endophytes to these grasses has recently led to their widespread investigation.

The endophytes are thought to have evolved from *Epichloë* spp. (Ascomycetes), the causative agents of "choke" disease of grasses, and share many similarities with them (An *et al.* 1992; Schardl *et al.* 1991; Schardl and Tsai 1992; Scott and Schardl 1993; Siegel *et al.* 1990). *Epichloë* spp. normally grow within the plant as an endophyte, but upon flowering choke of the inflorescences often occurs. Choke is the result of a mycelial growth (the stromata) on host leaf sheaths and inflorescences that prevents seed production on affected tillers. The stromata is the sexual structure of *Epichloë*, and produces asexual spores (conidia) which act as spermatia in sexual crosses (see Fig. 1 for life cycle of *Acremonium* and *Epichloë*). Following fertilisation of the stromata by conidia of the opposite mating type perithecia develop on this structure. The sexual cycle culminates with ejection of ascospores from mature perithecia, which germinate to give phialides and conidia. These conidia then undergo a microcyclic conidiation cycle of conidial germination, followed by growth and formation of further conidia. After the last cycle (typically the third) the mycelia are thought to infect the host via the stigmata, leaf stem stomata and/or ancillary buds. Following mycelial infection, meristematic tissue must become infected for survival of the fungus (Siegel and Schardl 1991).

The sexuality of *Epichloë* spp. is inversely proportional to that of the host grass. Some *Epichloë*-grass relationships result in complete sterilisation of the grass, limiting it to solely vegetative reproduction. For example the ryegrass choke pathogen, *Epichloë typhina*, nearly completely sterilises it's grass host (Siegel *et al.* 1987a). Conversely some associations very rarely result in choke and only a few inflorescences are infected, with the healthy inflorescences giving rise to seed infected with *Epichloë* (Siegel and Schardl 1991). Associations of *Epichloë* and fescue grasses typify this relationship (Leuchtmann *et al.* in press). In other associations choke has never been observed despite years of growth under a wide range of environmental conditions (Clay 1990, the symbiosis between *A. lolii* and perennial ryegrass). Hence, associations of grasses with *Epichloë* spp. and the related endophytes can be said to form an evolutionary continuum from mutualism to antagonism, and the degree of mutualism or antagonism is determined by the relative importance of the fungal sexual cycle.

1.2.1 Evolutionary Relationships

Despite wide variation in host species, geographic distribution, morphology, and the degree of antagonism, only a single species of *Epichloë, E. typhina*, was long recognised on pooid grasses (Kohlmeyer and Kohlmeyer 1974; Sampson 1933). However, recently a number of reproductively isolated mating populations of *Epichloë* have been identified from mating tests (Leuchtmann *et al.* in press; Schardl and Tsai 1992; White 1993). These mating populations correspond well to phylogenies derived from molecular data and are also distinguishable by morphology of fruiting structures, particularly size and disarticulation of ascospores (Leuchtmann *et al.* in press; Schardl *et al.* 1994; Schardl and Tsai 1992; Tsai *et al.* 1994). This has allowed the adoption of a biological species concept for the classification of *Epichloë* spp (Leuchtmann *et al.* in press; Schardl and Tsai 1992; White 1993). Currently four species of Epichloë have been identified from the pooid grasses: *E. typhina*; *E. baconii*; *E. clarkii*; and *E. festucae* (Leuchtmann *et al.* in press; White 1993). Other distinct mating populations have been identified, but are yet to be given specific epithets (Leuchtmann *et al.* in press).

E. typhina has a wide host range, including at least three genera in two tribes of the Pooideae: *Dactylis* and *Lolium* from the Poeae; and *Anthoxanthum* from the Aveneae being known hosts of *E. typhina* (Leuchtmann *et al.* in press). However, *E. typhina* from *Anthoxanthum odoratum* is genetically distant from *E. typhina* from *Lolium* and *Dactylis*. This is possibly due to temporal differences in flowering and stroma formation between *A. odoratum* and other *E. typhina* hosts, preventing interhostal mating (Leuchtmann *et al.* in press). In addition, it is unknown whether progeny from these interhostal matings would be infective on either host.

E. baconii has been identified on grasses of the genus *Agrostis*, while *E. clarkii* has been identified on *Holcus lanatus* (White 1993). Like *E. typhina*, *E. baconii* and *E. clarkii* are aggressive antagonists, typically completely sterilising their host grasses at the time of flowering.

E. festucae is found on fescue grasses (*Festuca* spp.). *E. festucae* apparently originated in Europe, where stromata production on infected grasses by these fungi is rare. Subsequently it has been introduced, presumably in infected seed, into a number of other geographical locations, including the U.S.A. In the U.S.A. *E. festucae* in infected grasses tends to form stromata more frequently than is observed in Europe.

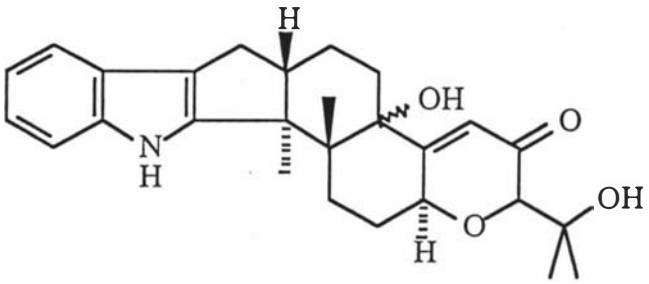
Unfortunately, classification of the asexual *Acremonium* endophytes is not possible with mating tests, and relies on other criteria. Christensen *et al.* (1993) used a number of criteria (morphology, alkaloid production, grass host, and isozyme

polymorphisms) to classify *Acremonium* endophytes from the grasses *L. perenne, F. arundinacea* and *F. pratensis* (meadow fescue). The endophytes were shown to possess considerable diversity. Three taxa of endophytes from tall fescue were identified. These were named *F. arundinacea* taxonomic grouping one (FaTG-1), FaTG-2 and FaTG-3. The isolates of FaTG-1 met the original definition of *A. coenophialum*, so were named this. Two taxa from perennial ryegrass, named *L. perenne* taxonomic grouping one (LpTG-1), which met the definition of *A. lolii,* and LpTG-2 were identified. Only one taxa from meadow fescue (*F. pratensis*) was identified, this taxa met the definition of *A. uncinatum.* These taxonomic groups were shown to correspond well with molecular phylogenies derived from nucleotide sequences of the ITS region of the rDNA repeat and the *tub*2 gene (Schardl *et al.* 1994; Tsai *et al.* 1994). Subsequently, Leuchtmann (1994) identified six taxa of endophytes from Fescue grasses, based on genetic identities calculated from isozyme data. These taxa included *A. uncinatum*, *A. coenophialum*, FaTG-2, *A. starrii*, a grouping which was probably *E. festucae* and a new unnamed group from *F. altissima.* This grass is a member of the primitive *Festuca* subgenera *Drymanthele,* and the endophyte isolated from this grass was very different, genetically and morphologically, to the other endophyte taxa identified from *Festuca* spp. This data suggests that individual grass species may possess endophytes which are specific to those species, and suggests, in *F. altissima* at least, that the endophytes may have a history of coevolution with their grass hosts.

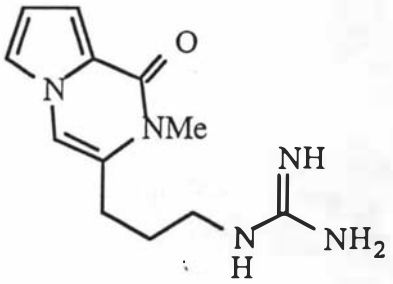
Thus the endophytes are a diverse group of fungi, and are found in a wide range of grass species. This diversity is not restricted to the sexual *Epichloë* spp., but is also found in their asexual relatives, the *Acremonium* spp. endophytic in pooid grasses. This diversity is potentially a problem for the classification of the *Acremonium* endophytes. However techniques which compare genetic relatedness (e.g isozyme, restriction fragment length polymorphisms or RFLPs, randomly amplified polymorphic DNA or RAPDs and sequence analysis) allow the evolutionary relationships of these organisms to be determined, and allow taxonomic relationships to be addressed. When integrated with other data (e.g. morphology) these have enabled classification of the endophytes into distinct taxa. Once these taxa are established the preferred method may be used to classify new isolates.

1.2.2 Biologically Active Secondary Metabolites

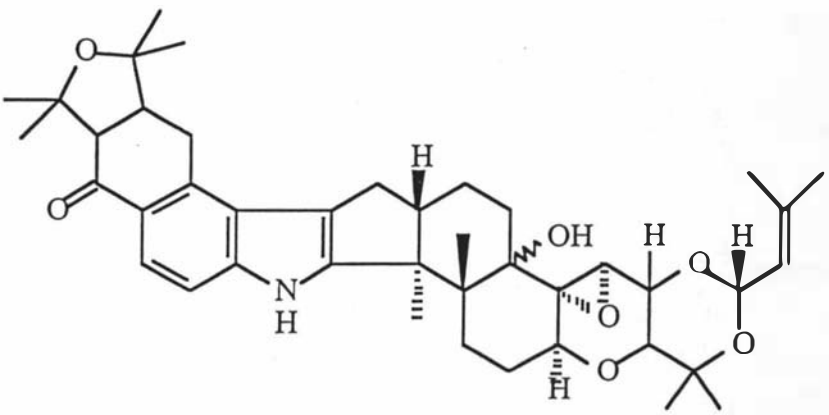
The symbioses between the endophytes and grasses are known to produce bioactive secondary metabolites, and these have been implicated in the protection of the grasses from herbivores. In particular high levels of the neurotoxin, lolitrem B (Fig. 2), and other indole diterpenoids, are produced in associations between perennial ryegrass and *Acremonium lolii*, the endophyte of perennial ryegrass (Gallagher *et al.* 1981;



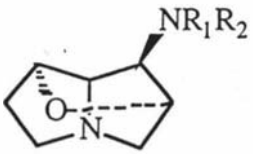
Paxilline



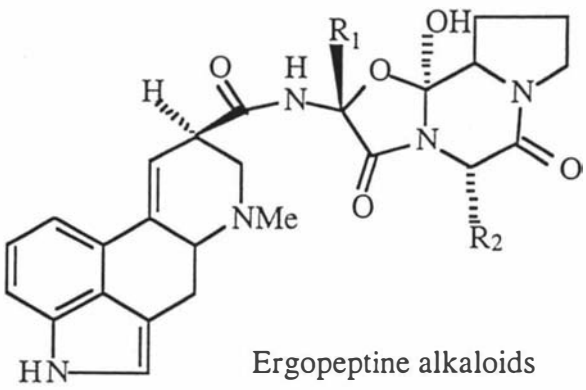
Peramine



Lolitrem B



Loline alkaloids



Ergopeptine alkaloids

N-Acetyl norloline: R_1 = H, R_2 = CH_3 CO

N-Formyl loline: R_1 = methyl, R_2 = CHO

N-Acetyl loline: R_1 = methyl, R_2 = CH_3 CO

Ergovaline: R_1 = methyl, R_2 = isopropyl

Ergotamine: R_1 = methyl, R_2 = benzyl

Fig. 2. Structures of biologically active secondary metabolites thought to play an important role in grass-endophyte associations. Courtesy of David McSweeney, Department of Chemistry and Biochemistry, Massey University.

Gallagher *et al.* 1982). These tremorgens have been implicated as causative agents of the neurological disorder ryegrass staggers, which is often exhibited by livestock grazing infected grass (Gallagher *et al.* 1982). Affected animals display muscle spasms and are hypersensitive to external stimuli. The principal site of action of these mycotoxins is thought to be the GABA receptor (Gant *et al.* 1987). Very little is known about the biochemical pathways giving rise to these compounds, although putative intermediates paxilline and lolitriol have been identified and the outlines of a biosynthetic pathway suggested (Miles *et al.* 1992). Paxilline (Fig. 2) is thought to be synthesised from tryptophan and mevalonic acid (Fig. 3) by way of a series of reactions with various modifications of geranyl geranyl pyrophosphate. Paxilline, a known tremorgen, is a fungal product, being produced erratically in culture by *A. lolii* (Penn *et al.* 1993), and constitutively in large quantities in culture by the fungus *Penicillium paxilli* (Cole *et al.* 1974; Weedon and Mantle 1987). Lolitrem has also been detected erratically in cultures of *A. lolii,* but less frequently than paxilline (Penn *et al.* 1993). This suggests that the host plant may play a role in the biosynthesis of lolitrem, probably indirectly, by regulating production of this compound. Not all associations of perennial ryegrass and endophytes result in production of indole diterpenoids, and it is not exclusive to these interactions (Christensen *et al.* 1993).

In the symbiosis between *A. coenophialum* and tall fescue, the ergopeptine alkaloids, particularly ergovaline (Fig. 2), have been implicated as the primary agents of fescue toxicosis (Raisbeck *et al.* 1991). This condition is characterised by reduced weight gain, decreased milk production, lower feed intake, rough hair-coat, excessive salivation, increased respiration rate and high temperature (Siegel *et al.* 1987b). These conditions are generally observed in summer months. Fescue foot is another toxicosis that may affect cattle which graze on endophyte-infected tall fescue. This malady is characterised by lameness, dry gangrene of the extremities, and in severe cases, loss of hooves (Siegel *et al.* 1987b). The biochemical mechanisms that are responsible for these symptoms are unknown. However, the ergot alkaloids are known to interact with the α-adreno and dopamine receptors and these compounds have vascoconstrictive effects (Porter 1994; Thompson and Garner 1994). These alkaloids are known fungal products, having been detected in fungal cultures (Porter *et al.* 1979; Porter *et al.* 1981). Ergovaline is thought to be synthesised from a tricyclic peptide and an ergolene ring synthesised from mevalonic acid and tryptophan (Fig. 3). Ergopeptines are found in a number of grass-endophyte interactions, including perennial ryegrass associations (Christensen *et al.* 1993).

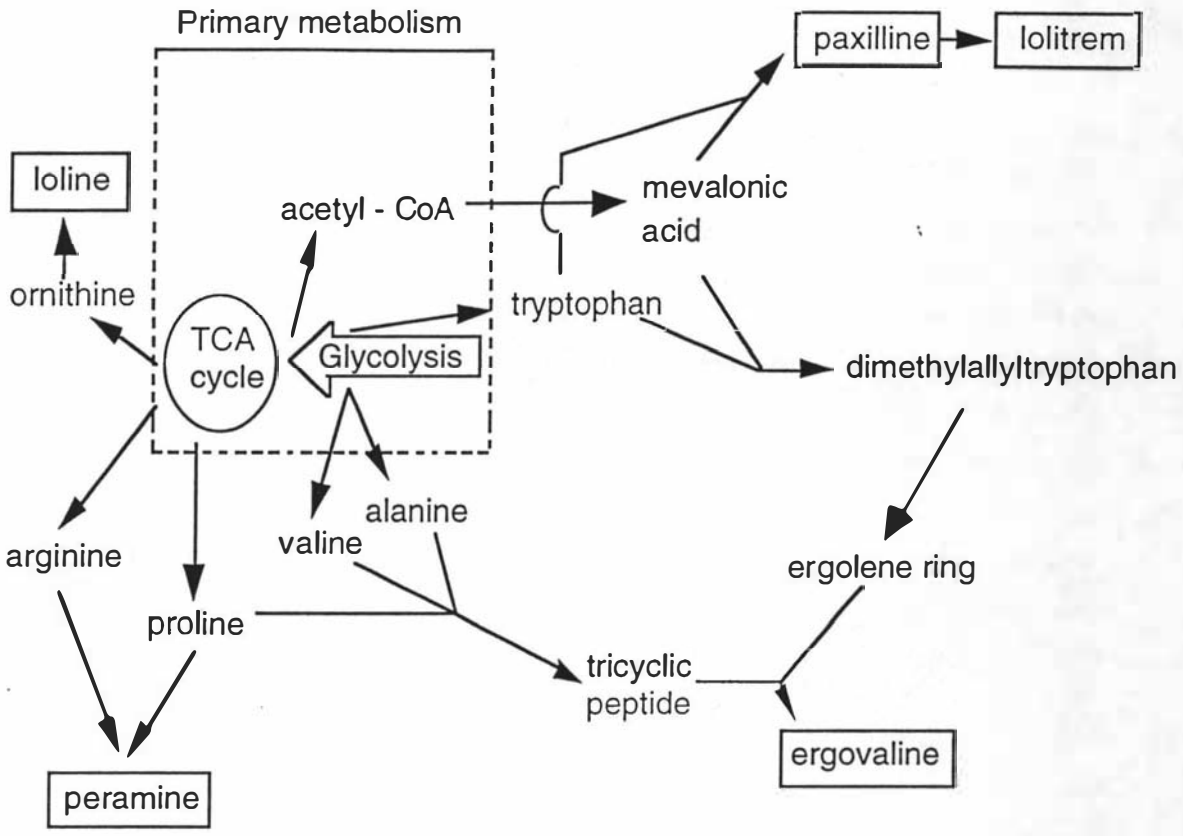


Fig. 3. Relationship of primary metabolism to the biologically active secondary metabolites (in boxes) thought to play an important role in grass-endophyte associations. Some probable precursors and intermediates are shown (Porter 1994). Courtesy of Terri Sargent, Department of Microbiology and Genetics, Massey University.

The saturated amino pyrrolizidines, or lolines (e.g. *N*-acetyl loline and *N*-formyl loline, Fig. 2), are the most abundant secondary metabolites that have been identified in the associations between tall fescue and *A. coenophialum* (Siegel *et al.* 1990; Siegel and Schardl 1991). Very high levels of these compounds have been detected in infected grasses (> 0.8% plant dry weight), but they have not yet been identified in fungal cultures or uninfected plants, hence it is uncertain whether they are solely fungal or plant in origin (Siegel and Schardl 1991). However, the production of these alkaloids is clearly dependent on the fungal strain (Siegel *et al.* 1990) and they are thought to be derived from ornithine (Fig. 3). These compounds are potent insecticides (Clay 1991), and may be very important in the symbiosis in this function, however they have not been implicated in fescue toxicoses (Scott and Schardl 1993). This insect toxicity is possibly associated with the ability of these compounds to disrupt physiological processes controlled by biogenic amines (Scott and Schardl 1993).

Peramine (Fig. 2), a pyrrolopyrazine alkaloid, is produced in many endophyte-grass associations, including *A. lolii*, *A. coenophialum* and *Epichloë* spp. in association with their host grasses (Schardl and Tsai 1992). It is a potent insect feeding deterrent, particularly against the Argentine stem weevil (*Listronotus bonariensis*), a major pest on grasses lacking this compound in New Zealand (Rowan and Gaynor 1986). The mechanism whereby peramine deters insects feeding on grass-endophyte associations producing this compound is unknown, but it is not known to be a mammalian toxin. Peramine has not been detected in fungal cultures or uninfected plants, so appears to be unique to the mutualistic association. It is thought to be synthesised from proline and arginine (Fig. 3).

All the secondary metabolites identified from endophyte-grass associations have activity against insects, though not all are mammalian toxins. For example Popay *et al.* (1990) measured the effects of peramine, lolitrem B and ergotamine on Argentine stem weevil. In choice tests peramine was found to deter adult feeding at less than 1 ppm. In no choice tests 10 ppm peramine significantly decreased feeding. In addition, 10 ppm peramine coated on grass stems decreased, but did not prevent, oviposition. Apart from deterring feeding, once larvae became established on their diet, larval development was not affected. However, lolitrem B at 5 ppm had no effect on adult weevil, but larvae were deterred from feeding, suffered reduced growth and had increased mortality. Ergotamine deterred adult feeding at less than 1 ppm, but had no effect on larval growth and survival.

Given the lack of detectable or consistent production of several of the alkaloids in fungal cultures and endophyte free grass, but detectable production in the

association, it seems likely that molecular interactions between the grass host and endophytic fungus occur, which results in higher expression levels of these compounds in the symbiotic association. In addition, *A. coenophialum* produces auxin in culture, although whether this relates to production and biological activity in the plant is unknown and difficult to assess (West 1994). Thiamine has been shown to be required for growth of *A. coenophialum* in culture, which suggests some nutritional dependence of the endophyte on the plant in the association (Kulkarni and Nielsen 1986).

The considerable diversity of secondary compounds produced in various relationships between endophytes and grasses may reflect genetic differences between these associations (i.e. the presence or absence of specific enzymes involved in these biochemical pathways), differential regulation in the expression of these pathways, or both. These pathways are obvious areas for investigations of the symbiotic relationship between the grasses and endophytes. However, a major problem is the lack of detectable production of many of these compounds in fungal cultures (Siegel and Schardl 1991). Ways around this problem are needed if progress is to be made. Methods currently under use or consideration include the use of model organisms that produce the desired secondary products in detectable quantities in culture. For example, *Penicillium paxilli* is an asexual fungus that readily produces large amounts of paxilline in culture (Mantle 1987). A transformation system has been established for this fungus (Itoh *et al.* 1994), and a mutagenesis program undertaken using a plasmid tagging strategy (Itoh and Scott 1994). A mutant in paxilline production has been identified and shown to possess a large deletion and translocation next to the site of plasmid insertion. This deletion or translocation has presumably disrupted the genes involved in paxilline biosynthesis. The next step is the cloning of the genes involved in production of this compound in *P. paxilli*, and making the step from there to *Acremonium.* In addition to model organisms, differential screening methods (Liang and Pardee 1992; Lisitsyn *et al.* 1993; Sargent and Dawid 1983) hold much promise for the isolation of genes involved in the symbiotic relationships. However, careful thought must be given to the use of such methods, and suitable material selected for screening for apparent differential expression.

Besides the isolation and characterisation of genes from these organisms, it is necessary to be able to modify the cloned genes *in vitro*, and then re-introduce them into the genome of the organism from which they have been cloned at the wild-type locus, in order to study the phenotypic effect of these changes on the symbiosis. For example, when a gene of uncertain function is cloned it is useful to be able to disrupt the open reading frame of the cloned gene, so it is no longer functional (i.e. create a null mutation of the gene). This null mutation is then used to replace the wild-type gene and the phenotype conferred by this null mutation assessed (Rothstein 1991, i.e. perform gene

knock-outs or disruptions). This should allow a function for the cloned gene to be determined. It may also be desirable to make more subtle changes to the gene (e.g. mutation of promoter sequences or hypothesised motifs of functional significance), replace the wild type gene with the mutated gene, and then study the phenotypic effects of these changes (Rothstein 1991, i.e. perform gene replacements). Several requirements need to be met if this technology is to be exploited. A transformation system is needed so it is possible to re-introduce cloned DNA into the genome of the endophytes. A high frequency transformation system has been developed for an *Acremonium* endophyte from perennial ryegrass (Murray *et al.* 1992), previously known as 187BB and since renamed Lp1 (Christensen *et al.* 1993). Transformation of filamentous fungi generally results in integration of the transforming DNA into the fungal genome. Integration can either occur at ectopic sites (i.e. sites with no or very low homology) or homologous sites (Fincham 1989). It is necessary that homologous integration occurs at a detectable frequency, so gene replacements or disruptions can be distinguished from the often more common ectopic integration events. Besides transformation systems, suitable selectable markers are needed if these gene manipulation systems are to be developed in this group of organisms. One marker which has been widely exploited in transformation systems of fungi, and has been used in gene disruption and replacement experiments, is the gene encoding orotidine-5'-monophosphate decarboxylase.

1.3 OROTIDINE-5'-MONOPHOSPHATE DECARBOXYLASE

The gene coding for orotidine-5'-monophosphate decarboxylase (OMPdecarboxylase) has been widely used in gene manipulation systems in filamentous fungi (Ballance *et al.* 1983; Ballance and Turner 1985; de Ruiter-Jacobs *et al.* 1989; Goosen *et al.* 1987; Gruber *et al.* 1990; Kronstad *et al.* 1989; Smit and Tudzynski 1992; Smith *et al.* 1991). The OMPdecarboxylase enzyme catalyses the final step in the pyrimidine biosynthetic pathway, the conversion of orotidine-5'-monophosphate (OMP) to uridine-5'-monophosphate (UMP, Fig. 4). The pyrimidine biosynthetic pathway converts glutamine, ATP and CO_2 through six enzyme catalysed steps into UMP, the precursor of all pyrimidine nucleotides (Fig. 4). A blockage at any stage in this pathway leads to a cessation of cellular growth, unless there is an exogenous supply of pyrimidine nucleotides, nucleosides or bases in the environment with mechanisms available for their uptake and salvage. The pyrimidine biosynthetic pathway is compartmentalised in the nucleus in the filamentous fungi (Radford *et al.* 1985). This aides channelling of pyrimidine biosynthesis, as the first step of this pathway is common to the arginine biosynthetic pathway, which in turn is compartmentalised in the mitochondria (Radford *et al.* 1985).

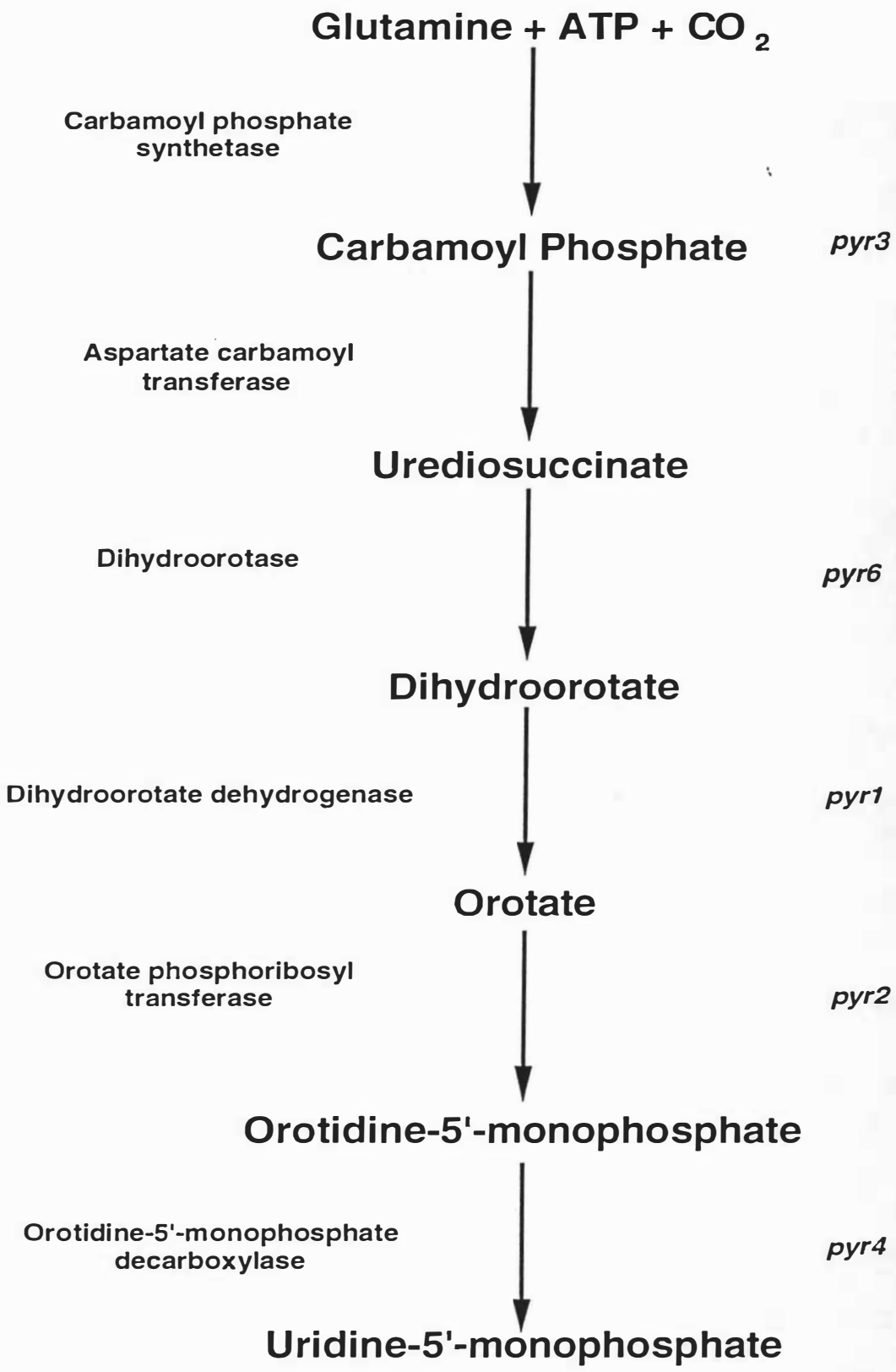


Fig. 4. The pyrimidine biosynthetic pathway, with the enzymes on the left, and the genes from *Neurospora* on the right (Radford *et al.* 1985).

The OMPdecarboxylase gene is a particularly useful genetic marker. Any loss of function mutants in this gene will be auxotrophs for pyrimidine (ie. unable to grow without an exogenous supply of pyrimidines such as uracil or uridine). This allows negative selection for OMPdecarboxylase mutants. In addition mutations at this locus can be positively selected on medium containing 5-fluoro orotic acid (5-FOA) based on their ability to grow in the presence of this usually toxic pyrimidine analogue (Boeke *et al.* 1984). The mechanism of 5-FOA toxicity is uncertain. Given the resistance of mutants in the orotate phosphoribosyl transferase and OMPdecarboxylase genes to 5-FOA, it seems likely 5-FOA is converted to 5-fluoro-dUMP by these two enzymes, followed by irreversible inhibition of thymidylate synthase through suicide inhibition (Boeke *et al.* 1984; Kalpaxis *et al.* 1991).

These selection systems have made the isolation of mutations at the OMPdecarboxylase gene locus relatively simple. In addition they have proven especially useful in 1-step and 2-step gene replacement experiments (Boeke *et al.* 1984; Rothstein 1991).

The nomenclature of the gene encoding the OMPdecarboxylase enzyme is confused. In *E. coli* it is referred to as the *pyr*F gene (Donovan and Kushner 1983), in *S. cerevisiae* it is *ura*3 (Rose *et al.* 1984), in the Plectomycetes and related Deuteromycetes it is *pyr*G (Oakley *et al.* 1987) and it is *pyr*4 in the Pyrenomycetes and related Deuteromycetes (Radford *et al.* 1985, e.g. *Neurospora*, *Epichloë* and *Acremonium*).

As a result of the powerful selection systems available for this gene and mutants thereof, as well as the availability of a number of clones of this gene from various fungi, it has been a popular target for fungal molecular geneticists to clone and/or mutate, generally with the intention of using it in transformation as a selectable marker. Thus, there is a wealth of information about the sequence and organisation of this gene, and this has given some interesting insights relating to its evolution (Radford 1993). In all the Pyrenomycetes for which sequence data on *pyr*4 is available there are no introns in the gene, and in addition there is a DNA insert of about 300 nucleotides corresponding to about 100 amino acids that is not found in the *pyr*G or *ura*3 genes (Newbury *et al.* 1986; Smith *et al.* 1991). However in the *pyr*G genes that have been sequenced from the Plectomycetes and related Deuteromycetes, i.e. *Aspergillus nidulans*, *A. niger* and *Penicillium chrysogenum* there is one intron between amino acids 62 and 63, the length of which is about 50 to 60 nucleotides (Oakley *et al.* 1987). In the Basidiomycete *Schizophyllum commune* and the Zygomycetes *Phycomyces blakesleeanus* and *Mucor circinelloides*, there are two short introns, at the same position in both fungi, one between

amino acid 62 and 63, as in the Plectomycetes, and one at amino acid 100 (Benito *et al.* 1992; Diaz-Minguez *et al.* 1990; Froeliger *et al.* 1989). This suggests a close evolutionary relationship between the Basidomycetes and Zygomycetes, and is supported by similarities in OMPdecarboxylase amino acid sequences. In addition all OMPdecarboxylases for which sequence information is available (ranging from *E. coli* and other prokaryotes to mammals such as human and mice) share a common conserved pentadecapeptide, which is possibly the OMP binding site (Vian and Penalva 1990).

1.4 AIMS AND BACKGROUND OF THIS STUDY

Given the wealth of information on the orotidine-5'-phosphate gene and its usefulness as a selectable marker, it was decided to clone the *pyr*4 gene from an *Acremonium* endophyte. This is an initial step into the molecular genetics of these organisms. *Acremonium* isolate 187BB, since renamed Lp1 (Christensen *et al.* 1993), which is a low lolitrem producing strain from perennial ryegrass, was chosen, as a high frequency transformation system had already been developed for this fungus (Murray *et al.* 1992), and a gene library was available (Appendix 1).

Thus, the initial aims of this investigation were:

(1) To clone and characterise the *pyr*4 gene from Lp1; and

(2) Use the cloned *pyr*4 gene to study the frequency with which gene disruptions occur in this endophyte.

Chapter 2.0 MATERIALS AND METHODS

2.1 FUNGAL AND BACTERIAL STRAINS, λ CLONES AND PLASMIDS

Fungal and bacterial strains, λ clones and plasmids used in this study are listed in Table 1.

2.2 GROWTH OF CULTURES

Acremonium and *Epichloë* cultures were grown at 22°C in potato dextrose broth or on potato dextrose agar (PDB or PDA, Section 2.3.1).

A. nidulans cultures were grown at 37°C on ACM, supplemented as needed with uracil (ACM+URA, Section 2.3.2) or with sucrose as an osmotic stabiliser for protoplast regeneration (ACM+S, Section 2.3.2) or both (ACM+URA+S).

For the growth of fungal cultures from which nucleic acid was to be extracted, 200 µl of a solution containing mycelia homogenised in MilliQ water (*Acremonium* and *Epichloë* isolates), or a spore suspension (*Aspergillus nidulans*) was spread on sterile cellophane discs placed on suitable media (Section 2.3). The cultures were incubated at the appropriate temperature until mycelial growth was visible (typically 5-7 days for *Acremonium* and overnight for *A. nidulans*). The mycelia was then harvested, by scraping it off the cellophane, and freeze dried.

For the growth of fungal cultures from which protoplasts were to be prepared two methods were employed. The method used for *A. nidulans* was the same as that employed for the growth of cultures for the extraction of nucleic acids. For *Acremonium*, 50 ml of PDB was inoculated with 200 µl of mycelia ground in water and the culture incubated at 22°C with shaking until growth was visible as mycelial pellets (typically 5-7 days).

E. coli cultures were grown at either 30°C or 37°C in LB broth or on LB agar (Section 2.3.3).

2.3 MEDIA

All media was sterilised at 121°C for 15 min prior to use. MilliQ water was used in all media. Liquid media was cooled to room temperature before addition of antibiotic(s) and inoculation. Solid media was cooled to approximately 50°C prior to antibiotic addition and pouring. Uninoculated plates were stored at 4°C.

Table 1 Strains, λ clones and plasmids.

Strain, λ clone or plasmid	Relevant characteristics	Source or reference
Fungal strains		
Acremonium sp. (= LpTG-2)		
Lp1	*Acremonium* sp. from *Lolium perenne*	(Christensen *et al.* 1993)
Lp2	*Acremonium* sp. from *Lolium perenne*	(Christensen *et al.* 1993)
ss1	Single spored isolate of Lp1	M. J. Christensen
ss5	Single spored isolate of Lp1	M. J. Christensen
ss10	Single spored isolate of Lp1	M. J. Christensen
ss12	Single spored isolate of Lp1	M. J. Christensen
ss16	Single spored isolate of Lp1	M. J. Christensen
ss20	Single spored isolate of Lp1	M. J. Christensen
Acremonium lolii (= LpTG-1)		
Lp5	*A. lolii* from *L. perenne*	(Christensen *et al.* 1993)
Lp6	*A. lolii* from *L. perenne*	(Christensen *et al.* 1993)
Lp7	*A. lolii* from *L. perenne*	(Christensen *et al.* 1993)
Lp9	*A. lolii* from *L. perenne*	(Christensen *et al.* 1993)
Lp19	*A. lolii* from *L. perenne*	(Christensen *et al.* 1993)
Acremonium uncinatum (= FpTG-1)		
Tf4	*A. uncinatum* from *Festuca pratensis*	(Christensen *et al.* 1993)
Acremonium coenophialum (= FaTG-1)		
Tf28	*A. coenophialum* from *Festuca arundinacea*	(Christensen *et al.* 1993)
Acremonium sp. (= FaTG-2)		
Tf15	*Acremonium* sp. from *F. arundinacea*	(Christensen *et al.* 1993)
Acremonium sp. (= FaTG-3)		
Tf18	*Acremonium* sp. from *F. arundinacea*	(Christensen *et al.* 1993)
Epichloë typhina (= MP-I)		
E8	*E. typhina* from *L. perenne*	(Byrd *et al.* 1990)
E. festucae		
E28	*E. festucae* from *Festuca longifolia*	(Leuchtmann *et al.* in press)
E32	*E. festucae* from *Festuca rubra* subsp. *commutata*	(Leuchtmann *et al.* in press)
Aspergillus nidulans		
1-85	*pyr*G89 *paba*A1 (y^+) *wA*3 (*qut*R16)	C. Roberts

Table 1 continued

Bacterial strains		
Escherichia coli		
LE392	*sup*E44 *sup*F58 *hsd*R514 *gal*K2 *gal*T22 *met*B1 *trp*R55 *lac*Y1	(Borck *et al.* 1976) (Murray *et al.* 1977)
MC1022	*ara*D Δ(*ara leu*) Δ(*lac*Z) M15 *gal*U *gal*K *str*A	(Casadaban and Cohen 1980)
XL1 - Blue	*sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A46 *thi rel*A1 lac$^-$ F' [*pro*AB$^+$ *lac*I^q *lac*ZΔM15 Tn10(tet^r)]	(Bullock *et al.* 1987)
PN1341	MC1022 / pMC1	This study
PN1343	MC1022 / pMC2	This study
PN1344	MC1022 / pMC3	This study
PN1358	MC1022 / pMC4	This study
PN1359	MC1022 / pMC5	This study
PN1402	XL1 - Blue / pMC7	This study
PN1420	XL1 - Blue / pMC11	This study
λ clones		
λEMBL3A	λ(*A*am32 *B*am1) *sbh*Iλ1^o *b*189 (polycloning site *int*29 *nin*L44 *trp*E polycloning site) KH54 *chi*C *sr*Iλ4^o *nin*5 *sr*Iλ5^o	(Frischauf *et al.* 1983)
λMC11	λEMBL3A clone containing *pyr*4-2 from Lp1	This study
λMC12	λEMBL3A clone containing *pyr*4-1 from Lp1	This study
λMC14	λEMBL3A clone containing *pyr*4-1 from Lp1	This study
λMC20	λEMBL3A clone containing *pyr*4-1 from Lp1	This study
Plasmids		
pAN8-1	5.9 kb Phleo^R Amp^R	(Mattern and Punt 1988)
pFB6	8.6 kb Amp^R containing *pyr*4 from *Neurospora crassa*	(Buxton and Radford 1983)
pGM32	4.4 kb Amp^R containing *pyr*4 from *N. crassa*	Greg May
pMC1	pUC118 containing a 0.8 kb *Bam*HI-*Sal*I fragment from λMC11	This study
pMC2	pUC118 containing a 2.2 kb *Bam*HI-*Eco*RI fragment from λMC12	This study
pMC3	pUC118 containing a 1.6 kb *Bam*HI fragment from λMC12	This study
pMC4	pUC118 containing a 2.4 kb *Bam*HI fragment from λMC11	This study
pMC5	pUC118 containing a 2.6 kb *Bam*HI-*Sal*I fragment from λMC11	This study
pMC7	pUC118 containing a 9.7 kb *Sal*I fragment from λMC14	This study
pMC11	pAN8-1 containing a 8.9 kb *Xba*I fragment from λMC11	This study
pRS4	pUC19 containing the *Claviceps purpurea pyr*4 on a 4.85 kb genomic *Sal*I fragment	(Smit and Tudzynski 1992)
pUC118	3.2 kb Amp^R	

2.3.1 Potato Dextrose Agar and Potato Dextrose Broth (PDA and PDB)

PDB contained 24.0 g of dehydrated potato dextrose broth (Difco) rehydrated in 1 litre of MilliQ water.

PDA was prepared by adding 15 g/l of agar (Davis) to PDB.

2.3.2 *Aspergillus* Complete Medium (ACM)

ACM contained (g/l): malt extract (Difco), 5.0; yeast extract (Difco), 2.5; $CuSO_4$, trace; agar, 20.0 (Davis). After autoclaving 25 ml/l of a sterile 40% (w/v) solution of glucose was added. For growth of *A. nidulans* strain 1-85, containing a *pyrG* mutation (Table 1), 2.2 g/l of uracil was added before autoclaving (ACM+URA). For protoplast regeneration 274 g/l of sucrose was added before autoclaving (ACM+S). Phleomycin (Phleo) was supplemented to a final concentration of 10 μg/ml where needed.

2.3.3 LB Media

LB media contained (g/l): tryptone (Difco), 10.0; yeast extract (Difco), 5.0; NaCl, 5.0. The pH was adjusted to 7.0 prior to autoclaving. For solid media, agar (Davis) was added to 15.0 g/l. Where needed, ampicillin was supplemented at a concentration of 100 μg/ml, isopropylthio-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside in dimethylformamide (X-gal) were both supplemented at a concentration of 40 μg/ml.

2.3.4 Top Agarose

Top agarose contained (g/l): tryptone (Difco), 10; NaCl, 5; agarose 15 (BDH), 8. This was cooled to 45-50°C following autoclaving and supplemented with $MgSO_4$ to 10 mM.

2.3.5 SOC medium

SOC medium (Dower *et al.* 1988) contained (g/l): tryptone (Difco), 20.0; yeast extract (Difco), 5; NaCl, 0.6; KCl, 0.2; $MgCl_2$, 0.95; $MgSO_4.7H_2O$, 2.5; and glucose, 3.6.

2.4 BUFFERS AND SOLUTIONS

2.4.1 1 x TBE Buffer contained 89 mM Tris-HCl, 2.5 mM Na_2EDTA, and 89 mM boric acid, pH 8.3.

2.4.2 STET Buffer contained 8% (w/v) sucrose, 5% (v/v) Triton X-100, 50 mM Na_2EDTA (pH 8.0) and 50 mM Tris-HCl (pH 8.0).

2.4.3 *Hae*III (Universal) Buffer was prepared at ten times the working concentration and contained 100 mM 2-mercaptoethanol, 60 mM Tris-HCl (pH 7.6) and 100 mM $MgCl_2$.

2.4.4 SDS Loading Buffer contained 1% (w/v) sodium dodecyl sulphate (SDS), 0.02% (w/v) bromophenol blue, 20% (w/v) sucrose, and 5 mM Na_2EDTA (pH 8 0).

2.4.5 TE Buffer (10 mM Tris-HCl/1 mM Na_2EDTA or 10 mM Tris-HCl/0.1 mM Na_2EDTA) was prepared to the required concentration from 1 M Tris-HCl (pH 8.0) and 250 mM Na_2EDTA (pH 8.0) stock solutions.

2.4.6 Hybridisation Buffer (Southern, 1975) contained (per litre): 50 ml 1 M Hepes (Sigma), pH 7.0; 150 ml 20 x SSC (Section 2.4.8); 6 ml phenol extracted herring sperm DNA (3 mg/ml, Sigma); 5 ml 20% (w/v) sodium dodecyl sulphate; 2 g Ficoll (Sigma 70); 2 ml *Escherichia coli* transfer RNA (10 mg/ml, Sigma); 2 g bovine serum albumin; 2 g polyvinylpyrrolidone(Sigma PVP-10); MilliQ water to 1 litre.

2.4.7 Tris-Equilibrated Phenol was prepared by melting solid phenol at 50°C. Hydroxyquinoline was added to a final concentration of 0.1% (w/v). An equal volume of 1 M Tris-HCl (pH 8.0) was added at room temperature and stirred for 15 min. The phenolic phase was retained and repeatedly washed with 1 M Tris-HCl (pH 8.0), until the pH of the phenolic phase was > 7.8. After equilibration the phenolic phase was retained and washed 2-3 times with 100 mM Tris-HCl (pH 8.0) containing 0.02% β-mercaptoethanol. The equilibrated phenol solution was stored under 100 mM Tris-HCl (pH 8.0) in a brown bottle at 4°C.

2.4.8 20 x SSC contained 3 M NaCl and 0.3 M sodium citrate.

2.4.9 2 x SSC was prepared by 10 fold dilution of 20 x SSC.

2.4.10 TES (10/1/100) contained 10 mM Tris-HCl (pH 8.0), 1 mM Na_2EDTA (pH 8.0) and 100 mM NaCl.

2.4.11 SM buffer contained (g/l): NaCl, 5.8 g; $MgSO_4.7H_2O$, 2.0 g; 1 M Tris-HCl (pH 7.5), 50 ml; 2% gelatin, 5 ml.

2.4.12 Acrylamide mix contained (g/l): urea, 480 g; acrylamide, 57 g; bis-acrylamide, 3 g. This mix was made up to < 900 ml and deionised with Amberlite MB-3 (Sigma), then filtered through a sintered glass funnel (porosity 1), 100 ml of 10 x sequencing TBE buffer (Section 2.4.16) was then added and the volume made up to 1 litre with MilliQ water.

2.4.13 OM Buffer contained 1.5 M $MgSO_4.7H_2O$, 10 mM Na_2HPO_4, and 100 mM $NaH_2PO_4.2H_2O$ added until the pH was 5.8.

2.4.14 STC buffer contained 1 M sorbitol, 50 mM Tris-HCl, and 50 mM $CaCl_2$, pH 8.0.

2.4.15 DNase free RNaseA was prepared from RNaseA at 10 mg/ml in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl, heated to 100°C for 15 min, allowed to cool slowly to room temperature, dispensed into aliquots and stored at -20°C.

2.4.16 10 x Sequencing TBE Buffer containing (g/l): Tris, 162 g; Na_2EDTA, 9.5 g; boric acid, 27.5 g. For running sequencing gels this buffer was diluted 10 x with MilliQ water.

2.5 DNA ISOLATION

2.5.1 Maxiprep DNA Isolation from Fungal Cultures

DNA was extracted from fungal cultures using the method of Byrd *et al.* (1990). In a pre-cooled mortar and pestle 0.5-1 g of freeze dried mycelium was ground to a fine powder under liquid nitrogen and then suspended in 10 ml of extraction buffer (150 mM Na_2EDTA, 50 mM Tris-HCl pH 8.0, 1% [w/v] sodium lauroyl sarcosine and 2 mg/ml proteinase K). The solution was centrifuged at 2 000 g for 10 min (all centrifugations were at 4°C), the supernatant incubated at 50°C for 20 min then phenol/chloroform extracted (Section 2.6, except no ethanol or isopropanol precipitation was carried out at this stage). The aqueous phase was then centrifuged at 25 000 g for 20 min. DNA was then ethanol precipitated (Section 2.7), resuspended in 500 µl of MilliQ water or TE and quantitated (Section 2.8.2 or 2.8.3).

2.5.2 Miniprep DNA Isolation from Fungal Cultures

Freeze dried mycelia (30-50 mg) was ground in a pre-cooled 1.5 ml microcentrifuge tube under liquid nitrogen. The powder was resuspended by vortexing

in 500 µl of extraction buffer (100 mM LiCl, 10 mM Na_2EDTA [pH 8.0], 10 mM Tris-HCl [pH 7.4] and 0.5% sodium dodecyl sulphate), phenol/chloroform extracted (Section 2.6), resuspended in MilliQ water or TE and quantitated (Section 2.8.2 or 2.8.3). This method was based on that of Yoder (1988).

2.5.3 Plasmid Isolation by the Rapid Boiling Method

The cells from 1.5 ml of an overnight *E. coli* LB broth culture, containing appropriate antibiotics and shaken at either 30°C or 37°C (Section 2.2), were pelleted by a 1 min centrifugation in a 1.5 ml microcentrifuge tube. The supernatant was drained and the pellet was resuspended in 350 µl of STET buffer (Section 2.4.2). Lysozyme (25 µl of a 10 mg/ml solution) was added and the tube was placed in a boiling water bath for 40 sec. The tube was then centrifuged for 10 min in a microcentrifuge and the resulting gelatinous pellet was removed with a sterile tooth pick. The DNA was precipitated by the addition of an equal volume of isopropanol. The contents were mixed by inversion and the tube was allowed to stand on ice for 10-20 min. The plasmid DNA was pelleted by centrifugation for 5 min in a microcentrifuge. The plasmid DNA pellet was washed once with 95% ethanol at room temperature, dried under a vacuum for 15 to 30 min prior to resuspension in 50 µl of MilliQ water or TE (10/0.1) and quantitated (Section 2.8.2 or 2.8.3). This method was based on that of Holmes and Quigley (1981).

2.5.4 Plasmid Isolation by a Small Scale Alkaline Lysis Method

The cells from 2 x 1.5 ml of an overnight *E. coli* LB broth culture, containing appropriate antibiotics and shaken at either 30°C or 37°C (Section 2.2), were pelleted by two 1 min centrifugations in a 1.5 ml microcentrifuge tube. The supernatant was drained and the pellets were resuspended in 100 µl of a solution containing 50 mM glucose, 25 mM Tris-HCl (pH 8.0) and 10 mM Na_2EDTA (pH 8.0). The mixture was incubated at room temperature for 5 min, 200 µl of a solution containing 0.2 M sodium hydroxide and 1% (w/v) SDS was added, mixed by rapid inversion several times, the mixture incubated on ice for 5 min and 150 µl of potassium acetate solution (29.44 g potassium acetate and 11.5 ml glacial acetic acid per 100 ml, pH 4.8) was added, mixed briefly by vortexing, and the mixture was incubated on ice for 5 min. The resulting precipitate was pelleted by centrifugation in a microcentrifuge for 3 min and the supernatant was transferred to a fresh 1.5 ml microcentrifuge tube. The DNA was then precipitated (Section 2.7). This method was based on that of Sambrook *et al.* (1989).

2.5.5 Plasmid Isolation by a Large Scale Alkaline Lysis Method

E. coli cells harbouring the required plasmid were grown in 500 ml of LB broth overnight with shaking at 37°C (1/100 inoculumn, Section 2.2). Cells were

harvested by centrifugation at 10 000 g for 10 min (all centrifugations were at 4°C), washed, by resuspending in 100 ml of TE (10/1, Section 2.4.5) and pelleting by centrifugation as before, then resuspended in 30 ml of a solution containing 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM Na_2EDTA (pH 8.0) and 150 mg of lysozyme. The suspension was incubated at room temperature for 10 min, then 60 ml of a solution containing 0.2 M sodium hydroxide and 1% (w/v) sodium lauroyl sarcosine was added, mixed by inversion, incubated for 10 min on ice, then 45 ml of a solution containing 29.44 g potassium acetate and 11.5 ml glacial acetic acid per 100 ml was added, mixed by inversion then incubated on ice for a further 10 min. The mixture was centrifuged for 10 min at 10 000 g and the DNA in the supernatant precipitated with isopropanol (Section 2.7). The DNA was resuspended in 7.5 ml of TE (10/1). This method was based on that of Ish-Horowitz and Burke (1981).

2.5.6 Purification of Plasmid DNA by Cesium Chloride-Ethidium Bromide Density Gradient

Cesium chloride (CsCl) was added to the plasmid DNA solution to be purified (prepared as in Section 2.5.5) in a ratio of 1.05 g/ml of DNA solution, 10 mg/ml of ethidium bromide was then added in a ratio of 75 µl/ml of DNA/CsCl solution, mixed well and left at 4°C overnight. The solution was then spun at 17 000 g for 10 min, the supernatant removed, the refractive index measured to check it was between n = 1.3860-1.3920, and if necessary adjusted. This solution was then ultracentrifuged for 5 h either at 70 K in a Beckman TLV-100 or 55 K in a Sorvall combi TV865. The band corresponding to ccc plasmid DNA was removed with an 18 gauge hypodermic needle and syringe and the ethidium bromide removed by extraction with SSC saturated isopropanol (prepared by stirring equal volumes of 20 x SSC and isopropanol for several hours). CsCl was then removed by dialysis against 1 litre of TES (10/1/100, Section 2.4.10) with 4 changes. Dialysis was performed with stirring at 4°C. The DNA solution was contained in dialysis tubing that had been pre-boiled for 10 min in 2% sodium bicarbonate, 1 mM Na_2EDTA (pH 8), rinsed well in MilliQ water, then boiled for 10 min in 1 mM Na_2EDTA. After dialysis DNA was quantitated as in Section 2.8.1.

2.5.7 Purification of Plasmid DNA by PEG Precipitation

To 7.5 ml of DNA prepared as in Section 2.5.5, 7.5 ml of ice-cold 5 M LiCl was added, mixed and centrifuged at 12 000 g for 10 mins (all centrifugations were at 4°C). DNA was precipitated from the supernatant with isopropanol (Section 2.7), resuspended in 500 µl of TE (10/1) containing DNase free RNaseA at 20 µg/ml (Section 2.4.15), incubated at room temperature for 30 min, 500 µl of 1.6 M NaCl containing 13% (w/v) PEG 8000 was then added, mixed well and the solution centrifuged. The

DNA pellet was resuspended in 400 µl of TE (10/1, Section 2.4.5), then extracted once with phenol then phenol/chloroform extracted as in Section 2.6 to remove any traces of PEG. The DNA was resuspended in 500 µl of TE (10/1), and then quantitated (Section 2.8.1). This method was based on that of Sambrook *et al.* (1989).

2.5.8 Purification of DNA from Phage λ

Phage were plated as in Section 2.13.1, except the phage were diluted to approximately 10^7 pfu/ml, giving approximately 10^6 pfu per plate. Once confluent lysis was nearly complete (ie. the LE392 lawn barely visible as small wispy patches on the plates), 5 ml of SM buffer was added to each plate and left overnight at 4°C. The resulting lysate was then collected, 5 ml of this lysate was centrifuged at 4 200 g for 10 min (all centrifugations were performed at 4°C) and DNase free RNaseA (Section 2.4.15) and DNase (Sigma) added to the supernatant at a concentration of 1 µg/ml, then incubated at 37°C for 30 min. After this step 5 ml of 20% (w/v) PEG 8000 in 2 M NaCl was added and the mixture incubated on ice for 1 h. The phage were pelleted by centrifugation at 4 200 g for 30 min and the pellet resuspended in 0.5 ml of SM buffer, 5 µl of 10% sodium dodecyl sulphate and 10 µl of 250 mM Na_2EDTA was added, and the solution incubated at 68°C for 15 min, then vortexed. An equal volume of phenol was added, the solution vortexed for 10 sec, left for 5 min then vortexed for another 10 sec. The aqueous phase was extracted from the phenolic phase then phenol/chloroform extracted (Section 2.6). The DNA was resuspended in 50 µl of TE (10/0.1, Section 2.4.5) containing 10 µg of DNase free RNaseA (Section 2.4.15) and quantitated (Section 2.8.2).

2.6 PURIFICATION OF DNA BY PHENOL/CHLOROFORM EXTRACTION

DNA samples were extracted with equal volumes of Tris-equilibrated phenol (Section 2.4.7) and chloroform, centrifuged (> 15 000 g), and the aqueous phase re-extracted until a clear interface between the aqueous and organic phases was obtained. Samples were then extracted once with two volumes of chloroform. The DNA was then precipitated by ethanol/isopropanol precipitation as described in Section 2.7. This method was as based on that of Sambrook *et al.* (1989).

2.7 PRECIPITATION OF DNA WITH ETHANOL OR ISOPROPANOL

One tenth volume of 3 M sodium acetate and either 2.5 volumes of 95% ethanol, or 0.6 volumes of isopropanol, were added to a tube containing DNA to be precipitated. The solution was mixed by inversion and the mixture was allowed to stand

on ice for at least 15 min after which time the DNA was pelleted by centrifugation at > 15 000 g for 5-10 min. The pellet was washed once with 70% ethanol and dried under vacuum until the alcohol had evaporated, before resuspension in MilliQ water or TE (Section 2.4.5). This method was based on that of Sambrook *et al.* (1989).

As noted above, 0.6 volumes of isopropanol was sometimes used in place of ethanol, however, ethanol was preferred as it is more volatile and solutes (e.g. NaCl) are less easily coprecipitated, thereby minimising coprecipitation of components that may prevent re-dissolution of the DNA.

2.8 DNA QUANTIFICATION

DNA was quantified by three methods, spectrophotometrically for pure solutions of high concentration (Section 2.8.1), fluorometrically for impure samples of high or low concentration (Section 2.8.2), or by intensity of ethidium bromide fluorescence for impure samples of very low concentration (Section 2.8.3).

2.8.1 Spectrophotometric Determination of DNA Concentration

Concentrated DNA solutions were diluted appropriately and the absorbance of the solutions in quartz cuvettes with a 1 cm light path was determined at both 260 nm and 280 nm. The reading at 260 nm allowed calculation of the concentration of nucleic acid present in the sample since an OD of 1 corresponds to approximately 50 µg/ml double stranded DNA. The ratio of readings at 260 nm and 280 nm (OD_{260}/OD_{280}) was used as an estimate of the DNA purity. Pure DNA has an OD_{260}/OD_{280} value of 1.8.

2.8.2 Fluorometric Quantitation of DNA

For impure DNA samples, or pure samples of low concentration, DNA was quantitated on a Hoefer Scientific TKO 100 Fluorometer. This method was suitable for quantitating down to 10 ng/µl and only 2 µl of DNA sample was needed for quantitation. DNA was quantitated in a dye solution containing 1 x TNE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA and 100 mM NaCl, pH 7.4) and 0.1 µg/ml Hoechst 33258. The scale of the fluorometer was set to 100 using 2 µl of 100 µg/ml calf thymus DNA added to 2 ml of the dye solution. Once the scale was reliably set, 2 µl of sample DNA was added to 2 ml of the dye solution and the resulting figure recorded as a concentration of ng/µl for the sample DNA solution.

2.8.3 Minigel Method for Determination of DNA Concentration

A sample from the DNA solution of interest was separated by electrophoresis through an agarose gel (Section 2.10) together with a series of standard DNA solutions

of known concentration. After the bromophenol blue dye front had migrated at least half way into the gel, the DNA was stained with ethidium bromide and photographed (Section 2.10). The intensity of fluorescence of the unknown DNA sample was then compared to that of the known DNA standards.

2.9 RESTRICTION ENDONUCLEASE DIGESTION OF DNA

Restriction endonuclease digests were carried out either in *Hae*III buffer (Section 2.4.3) with the salt concentration adjusted for each enzyme with a solution of 1 M NaCl in accordance with the manufacturers recommendations or with the manufacturers buffer supplied with the enzyme. DNA to be digested was quantitated (Section 2.8) and an excess of enzyme used to digest the DNA.

Digestions of plasmid and phage λ DNA were performed at the recommended temperature for 1 h and stored, on ice or at -20°C, while an aliquot was checked on an agarose gel (Section 2.10) to ensure completeness of digestion. In the event that a digestion was incomplete fresh enzyme was added and the mixture was incubated a further 1 h. Quantities of restriction endonucleases added were kept within suitable ranges to avoid star activity of the enzyme. In the event that the DNA was still not digested to completion, the DNA stock was further purified, by phenol/chloroform extraction (Section 2.6), and the digest repeated. Once complete, digestion was stopped by addition of 1/5 volume SDS loading buffer (Section 2.4.4). Digestion of fungal genomic DNA was performed in an identical fashion except digestion times were increased to a minimum of 3 h and a maximum of overnight incubation.

2.10 AGAROSE GEL ELECTROPHORESIS OF DNA

Horizontal agarose gel electrophoresis was performed either in a Mini-gel apparatus for 1-2 h or in a Biorad DNA Sub-Cell (150 x 200 mm gel bed) overnight. Agarose concentrations varied from 0.4% to 1.5%, except that for NuSieve agarose gel electrophoresis NuSieve agarose was used at a concentration of 4%. The appropriate quantity of agarose was added to 200 ml of TBE electrophoresis buffer (Section 2.4.1) and the agarose was melted in a microwave. After cooling to 50°C in a water bath, the gel was poured and allowed to set. DNA samples (with addition of 1/5 volume SDS loading buffer, Section 2.4.4) were loaded and the fragments were separated by electrophoresis at 80 V to 120 V (Minigels) or 30 V (Biorad Sub Cell). After electrophoresis, gels were stained with ethidium bromide, typically for 5-10 min, washed with MilliQ water, visualised on a UV transilluminator and photographed on Polaroid type 667 film.

DNA fragment sizes (in kilobases, kb) were determined, after agarose gel electrophoresis, by measuring the distance a fragment had migrated from the well in the gel. The molecular weight was then calculated by interpolation from a plot of the distance migrated in the same gel by suitable size (molecular weight) markers, against the logarithm of the molecular weight (kb or bp) of the size markers, or by use of the GELFRAGSIZER program (Gilbert 1990).

2.11 SUBCLONING

The process of subcloning typically involved recovery of DNA fragments from agarose gels (Section 2.11.1), ligation of DNA fragments into a suitable vector (2.11.2), electroporation of ligation mixtures into a suitable *E. coli* host (2.11.3), and then screening for recombinants by gel electrophoresis (Section 2.10) of diagnostic restriction digests (Section 2.9) of plasmid DNA isolated from transformants by the rapid boiling method (Section 2.5.3). When pUC118 was used as a vector for subcloning, blue/white selection (α-complementation) was employed to screen for putative recombinants in a suitable *E. coli* background (strains MC1022 or XL-1 Blue, see Table 1). In this case electroporated cells were plated onto LB medium containing ampicillin, IPTG and X-gal (Section 2.3.3) and white colonies were screened for recombinants as outlined above.

2.11.1 Recovery of DNA from Agarose Gels

DNA was recovered from SeaPlaque agarose gels (0.7% to 1.5% in TAE electrophoresis buffer [40 mM Tris-HCl, 20 mM glacial acetic acid and 2 mM Na_2EDTA, pH 8.2]) by phenol freeze extraction (Thuring *et al.* 1975). That is after gel electrophoresis to separate DNA fragments, the DNA fragment(s) of interest were visualised with a long wave UV lamp, excised from the gel with the minimum amount of excess agarose and placed in 1.5 ml microcentrifuge tubes. The agarose was melted at 65°C, covered with Tris-equilibrated phenol (Section 2.4.7), mixed, then frozen at -20°C for at least 2 h. The tube was then microcentrifuged for 10 min and the aqueous phase recovered and extracted with phenol/chloroform (Section 2.6).

2.11.2 DNA Ligations

2.11.2.1 CAP-Treatment of Vector DNA

Approximately 1.0 µg of vector DNA was digested to completion by the appropriate restriction endonuclease (Section 2.9), 1.0 µl of calf alkaline phosphatase (CAP, Boehringer) was then added and the mixture was incubated for 30 min at 37°C. The mixture was heated at 65°C for 5 min, then phenol/chloroform extracted (Section 2.6), and the precipitated DNA resuspended in MilliQ water.

2.11.2.2 Ligation

Ligation mixtures contained 2.0 µl of the manufacturers (New England Biolabs) 10 x ligation buffer, a 2-3 times molar excess of insert:vector (at least 20 ng of DNA insert and at least 20 ng of vector DNA), 1.0 µl of 1/10 or 1.0 µl of undiluted T4-DNA ligase (New England Biolabs), and MilliQ water (to 20 µl). For non-directional cloning the vector was pre-treated with calf alkaline phosphatase (Section 2.11.2.1). Ligation mixtures were left in a refrigerator or on the bench overnight.

To check that ligation had occurred, 2.0 µl of the ligation mix was removed prior to addition of T4-DNA ligase, 2.0 µl of SDS loading buffer (Section 2.4.4) was added and the sample was examined on an agarose gel (Section 2.10) alongside a 2.0 µl sample (with 2.0 µl of SDS sample buffer) removed after addition of T4 DNA ligase and overnight ligation.

2.11.3 Transformation of *E. coli* by Electroporation (Dower *et al.* 1988)

2.11.3.1 Preparation of Electro-Competent *E. coli* Cells

One litre of LB broth was inoculated (1/100) with the desired *E. coli* strain and grown at 37°C with vigorous shaking to mid-log phase (OD_{600} 0.5-1.0, about 3 h). The cells were chilled on ice for 20 min then harvested by centrifugation at 4 000 g for 10 min (all centrifugations were at 4°C). The cells were washed sequentially (by resuspension, centrifugation at 4 000 g to pellet then draining supernatant) in ice cold water (1 litre followed by 500 ml) and then ice cold 10% glycerol (20 ml then finally resuspended in 4 ml). Cell suspensions were stored at -70°C in 200 µl aliquots.

2.11.3.2 Electroporation

Electro-competent *E. coli* cells (Section 2.11.3.1) were thawed gently at room temperature, divided into 40 µl aliquots in ice cold microcentrifuge tubes, 1-2 µl of

DNA added (generally ligations from Section 2.11.2.2), mixed and the DNA/cell mixture left on ice for 1 min. The gene pulser (Biorad) was set to 25 μF and 2.5 kV and the pulse controller to 200 Ω resistance, in parallel with the sample chamber. The mixture of DNA and cells was transferred to an ice-cold 0.2 cm cuvette, shaken to the bottom, then pulsed at the above settings and the time constant checked. When a time constant of 4-5 msec was obtained the cells were immediately resuspended in 1 ml of SOC medium (Section 2.3.5) and incubated at 37°C for 1 h to aid recovery of transformed *E. coli*. A positive (typically 2 ng of pUC118) and negative (water only) control was always employed. Cells were plated at suitable dilutions onto selective LB plates (Section 2.3.3).

2.12 SOUTHERN BLOTTING AND HYBRIDISATION

2.12.1 Southern (Capillary) Blotting

DNA to be transferred to the Nylon membrane was separated by overnight gel electrophoresis, stained, visualised and photographed as describe in Section 2.10. The gel dimensions were measured after removal of the gel portion above the wells.

The gel was placed in a dish containing 250 mM HCl and gently agitated for 15 min. The HCl was poured off and the gel was immersed in 500 mM NaOH, 500 mM NaCl, with gentle agitation, for 30 min. The NaOH, NaCl solution was drained and the gel was immersed in 500 mM Tris (pH 7.4), 2 M NaCl, with gentle agitation, for 15 min. The gel was finally washed for 2 min in 2 x SSC (Section 2.4.9). The gel was washed in two changes of MilliQ water between all changes of solutions.

While the gel was being treated, a plastic trough with wells at each end was prepared by placing two sheets of Whatman 3MM chromatography paper soaked in 20 x SSC (Section 2.4.8) in the trough such that the ends of the paper projected into the wells. The wells were then filled with 20 x SSC to just below the horizontal surface of the paper between the wells. A sheet of Gladwrap was placed over the trough and pressed flat. A grid 2 mm less than the gel size was marked on the Gladwrap and removed. The treated gel was placed, inverted, over the grid such that the edges of the gel overlapped the edges of the grid. A piece of nylon membrane (Hybond-N, Amersham), cut to 2 mm greater than the gel size and pre-soaked in 2 x SSC, was placed over the gel ensuring that no air bubbles were present. Two pieces of Whatman 3MM chromatography paper, cut 2 mm less than the gel size and pre-soaked in 2 x SSC, were placed over the membrane. Two identically sized pieces of Whatman 3MM chromatography paper (unsoaked) were placed upon the two soaked pieces of 3MM paper. A stack of paper towels approximately 50 mm deep was placed upon the chromatography paper, followed by a

flat metal or plastic tray and a weight sufficient to keep the entire stack flat. After overnight transfer, the apparatus was disassembled and the membrane was washed for 5 min in 2 x SSC, then baked under vacuum at 80°C for 2 h. This method was based on the method of Southern (1975).

2.12.2 Preparation of [α-^{32}P]dCTP-Labelled Probe with the Ready-To-Go DNA Labelling Kit

DNA to be labelled (25-50 ng), in a 45 µl volume, was denatured in a boiling water bath for 3 min then immediately placed on ice for 2 min to cool. The denatured DNA solution was then added to the tube containing the Ready-To-Go (Promega) reagent mix, 3-5 µl of [α-^{32}P]dCTP (3000 Ci/mmol) was added, and if needed, MilliQ water to a final volume of 50 µl. The reaction was mixed by gently tapping the tube, spun briefly in a microcentrifuge and incubated at 37°C for 15 min.

Unincorporated nucleotides were separated from labelled DNA on a minispin Sephadex G-50 column (Section 2.12.4) equilibrated with TES (10/1/100, Section 2.4.10).

2.12.3 Preparation of [α-^{32}P]dCTP-Labelled Probe DNA with Individual Reagents

DNA to be labelled was digested for 30 min at 37°C with *Hae*III in a 25 µl reaction mixture containing 200-250 ng of DNA, 2.5 µl of *Hae*III buffer (Section 2.4.3), and MilliQ water to 25 µl, 4.0 µl of random primers (100 µg) were then added and the mixture was boiled for 2 min then chilled rapidly on ice. The following reagents were then added in the listed order: 2.5 µl of MilliQ water, 1.5 µl of *Hae*III buffer, 1.0 µl of 20 mM dGTP; 1.0 µl of 20 mM dATP; 1.0 of µl 20 mM dTTP; 3.0 µl of [α-^{32}P] dCTP (3000 Ci/mmol); and 1.0 µl of DNA polymerase I (Klenow fragment, Boehringer Mannheim). The reagents were mixed by gently tapping the reaction tube, spun briefly in a microcentrifuge and incubated at 37°C for 30 min to 2 h. The reaction was stopped by the addition of 3 µl of 250 mM Na_2EDTA (pH 8.0).

Unincorporated nucleotides were separated from labelled DNA on a minispin Sephadex G-50 column (Section 2.12.4) equilibrated with TES (10/1/100, Section 2.4.10).

2.12.4 Separation of Unincorporated Nucleotides by Minispin Column Chromatography

Minispin columns were constructed by plugging the bottom of a 1 ml plastic, disposable, Terumo, Tuberculin syringe with siliconised glass wool. The syringe was filled with Sephadex G-50 resin, equilibrated in TES (10/1/100, Section 2.4.10). Additional resin was added until the syringe was full to the 1 ml mark. The end of the syringe was inserted into the perforated cap of a 1.5 ml microcentrifuge tube. The assembly was inserted into a disposable plastic tube and centrifuged at speed 3 in a BTL bench centrifuge (approximately 1 500 g) for 4 min at room temperature in a swinging bucket rotor (all subsequent centrifugation steps described were also at the same speed and duration). Additional resin was added until the volume of resin in the syringe, after centrifugation, was unchanged at approximately 0.9 ml, 100 µl of TES (10/1/100, Section 2.4.10) was then added to the column, which was recentrifuged, this step was repeated twice. The radiolabelled DNA was added to the column in 100 µl of TES (10/1/100) and recentrifuged into an empty 1.5 ml microcentrifuge tube.

2.12.5 Hybridisation of Probe DNA to Southern Blots

The Southern blot (Section 2.12.1) to be probed was prehybridised for at least 2 h at 65°C (unless otherwise indicated) in hybridisation buffer (Section 2.4.6) in a sealed glass tube. After prehybridisation, all but approximately 5 ml of the hybridisation buffer was poured off, and boiled [α-^{32}P]dCTP-labelled probe was then added (Section 2.12.2 or 2.12.3).

After overnight hybridisation at 65°C (unless otherwise indicated), the filter was removed and washed as determined in Section 3.1.2 (unless otherwise indicated). The washed filter was then wrapped in Gladwrap while still damp, and exposed, in the presence of a Cronex intensifying screen, to a sheet of Fuji Medical X-ray film in a X-ray cassette. After exposure for a suitable period of time at -70°C, the film was developed in a Kodak X-Omat automatic processor.

2.12.6 Stripping Hybridised DNA off Southern Blots

A boiling solution of 0.1% sodium dodecyl sulphate was poured over the filter to be stripped, and the filter was gently shaken in this solution overnight while the solution cooled to room temperature. The filter was then checked by autoradiography (as outlined in Section 2.12.5) to ensure that stripping of the filter had occurred. If stripping was incomplete this process was repeated.

2.13 LIBRARY SCREENING BY PLAQUE HYBRIDISATION

Recombinant phage were screened by plating phage (Section 2.13.1), taking filter lifts off the resulting plaques (Section 2.13.2), and hybridisation of an appropriate probe to the filters (Section 2.13.3). Plugs containing positive plaques were stored overnight in SM buffer (Section 2.4.11) containing a drop of chloroform. The phage from these plaques were purified twice more by screening as outlined above except only 30-300 phage per plate were plated on the second and third screens. DNA was then extracted from positive plaques (Section 2.5.8). This method was based on that of Sambrook *et al.* (1989).

2.13.1 Plating Phage λ

E. coli strain LE392 was used as a host for phage λ. The phage λ population to be screened was diluted to $2x10^4$ pfu/ml in SM buffer (Section 2.4.11) and 100 µl of phage mixed with 100 µl of an overnight culture of LE392 grown in LB (Section 2.3.3) supplemented with $MgSO_4.7H_2O$ (10 mM) and maltose at 0.2% (w/v). The phage LE392 mixture was incubated at 37°C for 30 min, then added to 3 ml of top agarose (Section 2.3.4) at 45-50°C, and poured onto LB agar plates (8 cm diameter). The top agarose was allowed to set, then the plates were incubated at 37°C for about 6 h (until small plaques were visible). Plates were then stored at 4°C overnight.

2.13.2 Filter Lifts

Filters (Millipore HATF 08250) were placed on the LE392/phage lawn (from Section 2.13.1), marked asymmetrically so the position of the filters was known for later reference and left on the plate for 1 min once the filter was completely wet. The filters were then removed and placed DNA side up on 3 layers of 3 MM soaked in 500 mM NaOH, 500 mM NaCl (2 min) then 500 mM Tris-HCl (pH 7.4), 2 M NaCl (5 min) and finally 2 x SSC (2 min). The filters were then air dried and baked in a vacuum oven at 80°C for 2 h. Two lifts per plate were performed, on the second lift filters were left on the plates for 1 min 30 sec.

2.13.3 Hybridisation of phage λ DNA to [α-^{32}P]dCTP labelled DNA probe

Labelling of the DNA to be used as a probe, hybridisation of filters to labelled DNA and autoradiography was performed as detailed in Sections 2.12.2-2.12.5 except hybridisations were performed in plastic pots approximately 10 cm in diameter (up to 15 filters/pot). Positive plaques were identified as signals on autoradiographs in identical positions on duplicate filters. The positions of the positive plaques on the

LE392 lawn were established by alignment of the asymmetric markings on the plates and filters with the autoradiograph, and plaques corresponding to signals on the autoradiographs were picked. Positive plaques were picked with the pipette tip of a 1 ml Gilson autopipettor from which the end 5-10 mm had been removed.

2.14 RESTRICTION MAPPING λ CLONES WITH A λ MAPPING SYSTEM

A λ mapping system from Amersham (RPN1721) was used to map DNA from λ clones. Reagents described here were supplied with this kit.

2.14.1 5' End Labelling of Oligonucleotides

In a microcentrifuge tube 4.5 µl of an oligonucleotide to be labelled (1 pmol/µl of ON-L or ON-R, complementary to the 12 bp 5' overhangs at the ends of the λ arms), 3 µl of [γ^{32}P]ATP (10 mCi/ml, >5000 Ci/mmol), 1.5 µl of 10 x kinase buffer (1 M Tris-HCl, 100 mM $MgCl_2.6H_20$, 70 mM DTT, pH 8.0), 1 µl of polynucleotide kinase (3 units) and 5 µl of MilliQ water was incubated at 37°C for 1 h, then heated to 65°C for 3 min. This reaction was then diluted by placing 2 µl in a solution containing 125 µl of TE (10/1), 75 µl of gel loading dye solution (4 g sucrose, 160 mg Tris-HCl, 370 mg Na_2EDTA, 4 mg bromophenol blue in 10 ml of MilliQ water) and 50 µl of 1 M NaCl.

2.14.2 Partial Restriction Digestion of λ Clones

λ clone DNA (500 ng, Section 2.5.8) was digested with 0.2 units of *Bam*HI (BRL) or 0.1 units of *Sal*I (BRL) in 30 µl of the manufacturers 1 x buffer, at 20°C for 45 min. At 15 min intervals 10 µl aliquots were added to a stop tube containing 3 µl of 100 mM Na_2EDTA (pH 8.0) on ice, so that after 45 min the stop tube contained the 30 µl digestion and 3 µl of Na_2EDTA.

2.14.3 Hybridisation of End Labelled Primers to Partially Digested DNA and Separation by Gel Electrophoresis

In a microcentrifuge tube 5 µl of diluted 5' end labelled ON-L (Section 2.14.1) was added to 11 µl of partially digested λ clone DNA (Section 2.14.2), and in another tube 5 µl of diluted 5' end labelled ON-R was added to the remaining 22 µl of partially digested DNA. In another two microcentrifuge tubes 1 µl of the size markers provided with the λ mapping system was diluted in 9 µl of MilliQ water and 5 µl of diluted 5' end labelled ON-L or ON-R (Section 2.14.1) was added. These mixtures were heated to 70°C for 3 min then incubated at 42°C for 30 min, then loaded onto a 0.4% agarose gel in 1 x TBE buffer and separated by gel electrophoresis in a Biorad DNA Sub-

Cell (150x200 mm gel bed) at 2V/cm for 24 h. The gel was then dried and exposed, in the presence of a Cronex intensifying screen, to a sheet of Fuji Medical X-ray film in a X-ray cassette. After exposure for a suitable period of time at -70°C, the film was developed in a Kodak X-Omat automatic processor.

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

For n PCR reactions a cocktail for n+1 reactions was prepared on ice. A cocktail for 1 reaction contained: 5 µl of 10 x Taq buffer (Boehringer, containing 1.5 mM $MgCl_2$); 2 µl of 1.25 mM dNTPs; 10 pmol of "forward" primer; 10 pmol of "reverse" primer; 2 units of Taq DNA polymerase (Boehringer); and MilliQ water to 40 µl. The sequences of the "forward" and "reverse" primers used were dependent on the sequence being amplified. The cocktail was aliquoted out into 40 µl quantities in 0.5 ml microcentrifuge tubes for use in a Perkin-Elmer Cetus DNA thermal cycler or 0.2 ml strip tubes or microtitre dishes for use in a Corbett FTS-960 thermal cycler. A quantity of DNA corresponding to 10^5-10^6 molecules thought to contain the sequence to be amplified, in a volume of 10 µl, was added to the appropriate tube and mixed gently. A negative control containing water only was always included in each PCR run, this was prepared as the last reaction in each set. If 0.5 ml microcentrifuge tubes were being used then the reactions were overlaid with a drop of mineral oil. The reaction vessels were placed in the appropriate thermal cycler preheated to 94°C and after an initial 2 min melt at 94°C subjected to 35 cycles of 94°C for 45 sec, 58°C for 45 sec and 72°C for 60 sec. After the 35 cycles were complete the reactions were incubated at 72°C for 5 min then stored at 4°C or -20°C. Reactions were checked on a minigel (Section 2.10).

2.16 DNA SEQUENCING

Two different sequencing protocols were employed, the *fmol* kit from Promega and Sequenase Version 2.0 from USB. Both methods are based on the dideoxy-mediated chain termination method of Sanger (1977). The major difference between the two methods was in the DNA polymerase used. The *fmol* system used a modified form of Taq DNA polymerase, this allowed sequencing reactions to be performed at high temperatures (chain extension/termination reactions were performed at 72°C), and thermal cycling of the sequencing reactions, allowing multiple cycles of template melting, primer annealing and extension, hence only a very small amount of target DNA was needed before readable sequence was obtained.

2.16.1 Purification of PCR Products for Sequencing

PCR products were sequenced solely with the *fmol* kit, after the PCR product to be sequenced was purified with the Wizard PCR Preps DNA purification system. Completed PCR product (30-50 µl, Section 2.15) was added to 100 µl of Wizard PCR Preps Direct Purification Buffer, this mixture was vortexed briefly, 1 ml of Wizard PCR Preps DNA Purification Resin added and the mixture vortexed briefly three times over a 1 min period. This mixture was then loaded onto a Wizard PCR Preps Minicolumn using a 3 ml syringe with a Leur tip, the Minicolumn washed with 2 ml of 80% isopropanol, centrifuged briefly to dry and the DNA washed off the Minicolumn by centrifuging 50 µl of MilliQ water through the column at 12 000 g.

2.16.2 *fmol* DNA Sequencing Protocol

For each set of sequencing reactions four 0.5 ml microcentrifuge tubes (for use with the Perkin-Elmer Cetus DNA thermal cycler) or four 0.2 ml strip tubes or microtitre dish wells (for use with the Corbett FTS-960 thermal cycler) were labelled A, G, C and T and 2 µl of the appropriate d/ddNTP mix was added to each tube and the tubes left on ice. For each set of sequencing reactions a cocktail was made as follows: Template DNA (500 fmol of plasmid DNA [Section 2.5.6 or 2.5.7] or 40 fmol of PCR product [Section 2.16.1]); 3.0 pmol of primer; 0.5 µl of [α-^{35}S]dATP (>1000 Ci/mmol); 5 µl of 5 x fmol sequencing buffer; 1 µl (5 units) of sequencing grade TAQ polymerase and sterile MilliQ water to 17 µl, 4 µl of this enzyme/primer/template cocktail was then added to each tube containing the d/ddNTPs and mixed gently. If the Perkin-Elmer Cetus thermal cycler was being used then one drop of mineral oil was added to the reaction, and the tube briefly centrifuged. The reaction vessels were placed in the appropriate thermal cycler preheated to 94°C, and after an initial 2 min melt at 94°C, subjected to 30-60 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec. After the cycles were complete 3 µl of *fmol* sequencing stop solution was added to the reactions and the reactions stored at -20°C.

2.16.3 Sequenase Version 2.0 Sequencing Protocol

A freshly prepared solution of 2 M NaOH, 2 mM Na_2EDTA (2 µl) was added to approximately 5 µg of plasmid DNA (in the size range 3-6 kb) in a volume of 20 µl and incubated at room temperature for 5 min. Then 8 µl of 1 M Tris-HCl, pH 4.5 and 3 µl of 3 M sodium acetate were sequentially added to the DNA on ice, the solution mixed well, and 75 µl of ice-cold 95% ethanol added. The DNA was pelleted by centrifugation at 4°C for 10 min, the pellet washed with 200 µl of 70% ethanol and dried under vacuum, 1 µl of the appropriate sequencing primer, 2 µl of the 5 x sequenase

sequencing buffer and 7 µl of MilliQ water were then added to the dried DNA, the solution mixed and the primer left to anneal at 37°C for 30 min. While the annealing mixes were incubating the chain termination mixes were set up on a microtitre plate: one set of the four termination mixes per sequencing reaction; 2.5 µl of the appropriate d/ddNTP mix per well. After the 30 min incubation of the annealing mixes 1.0 µl of 0.1 M dithiothreitol, 2.0 µl of labelling mix diluted 5x in MilliQ water, 0.5 µl of [α-^{35}S]dATP and 2.0 µl of Sequenase diluted 8x in TE (pH 7.4) were added to the annealing mix. These labelling reactions were incubated at room temp for 5 min, then 3.5 µl of the labelling reactions were transferred to each of the four termination mixes on the microtitre dish and incubated at 37°C for 5 min. Sequenase stop solution (4 µl) was then added to each well and the reactions stored at -20°C.

2.16.4 PAGE Gel Electrophoresis of Sequencing Reactions

Sequencing reactions (Section 2.16.2 or 2.16.3) were separated by polyacrylamide gel electrophoresis (PAGE). Sequencing gels were poured with 60 ml of acrylamide mix (Section 2.4.12) containing 36 µl of TEMED and 360 µl of 10% (w/v) ammonium persulphate. Once gels had set they were pre-run for 15-60 min with constant power (65 W) in 1 x TBE sequencing buffer (Section 2.4.16). Sequencing reactions were then denatured at 75°C for 2 min and 3 µl loaded onto the sequencing gel. These reactions were run until the second dye front (the xylene cyanol FF) had run off the end of the gel (long runs, typically about 4 h) then the same sets of reactions were loaded onto the gel and run until the first dye front from these reactions (the bromophenol blue) had run off the gel (short runs, typically 2 h). The gel was then disassembled, fixed in a solution containing 10% acetic acid, 10% ethanol for 30 min, dried for 35 min under vacuum at 80°C then autoradiographed overnight.

2.17 TECHNIQUE FOR WORKING WITH RNA

All RNA work was performed in either acid washed glassware which had been baked in a dry air oven at 180°C for at least 2 h or with previously untouched disposable plastic labware (tips and eppendorfs). All equipment was handled with clean disposable gloves and all experimental work was performed wearing these. Plastic labware was stored in acid washed, baked glass beakers and sterilised by autoclaving at 121°C for 15 min separate from other reagents and labware. One set of Gilson pipettors was used exclusively for RNA work. Solutions were prepared from previously unopened stocks of reagents. All reagents (except those containing Tris-HCl, Na_2EDTA or MOPS) were treated with 0.1% diethyl pyrocarbonate (DEPC), incubated overnight at 37°C then the DEPC was removed by autoclaving twice. Reagents and labware for RNA work was stored separately from that used for other purposes.

2.18 ISOLATION OF RNA FROM FUNGAL CULTURES

The precautions given in Section 2.17 were used throughout. Approximately 200 mg of fresh mycelia (Section 2.2) was ground to a powder under liquid nitrogen in a pre-cooled mortar and pestle. The powder was transferred to 1 ml of 80°C phenol extraction buffer (containing 100 mM Tris-HCl, pH 8.0; 10 mM Na_2EDTA; and 1% sodium dodecyl sulphate, mixed with 1 volume of Tris equilibrated phenol, Section 2.4.7) and homogenised by vortexing. The solution was incubated at 80°C for 5 min then an equal volume of chloroform/isoamyl alcohol (24:1) added and the solution phenol/chloroform extracted (as in Section 2.6 except chloroform containing isoamyl alcohol in a ratio of 24:1 was used and the ethanol/isopropanol precipitation step was omitted). Half a volume of 8 M LiCl was added, vortexed to mix and incubated at -20°C overnight. RNA was then pelleted by centrifugation at 17 000 g for 30 min (all centrifugations were performed at 4°C). The RNA was washed with 1 volume of 2 M LiCl in 50 mM Na_2EDTA and repelleted as above. The pellet was washed twice with 3 M sodium acetate (pH 5.5) then resuspended in 100 µl of TE (10/1). This method was based on that of Shirzadegan *et al.* (1991).

2.19 NORTHERN BLOTTING OF RNA AND HYBRIDISATION TO [α-^{32}P]dCTP-LABELLED PROBES

This method was based on that of Bradshaw and Pillar (1992). The precautions given in Section 2.17 were taken throughout.

2.19.1 Glyoxylation of RNA

All steps of this process were performed under autoclaved light mineral oil (Sigma M-3516). A mixture of 40% deionised glyoxal (Sigma, G-3140, deionised under light mineral oil on a mixed bed resin [Biorad AG 501-X8] and stored in aliquots at -80°C), 20 x MOPS buffer (400 mM 3-[*N*-morpholino] propane-sulfonic acid [MOPS], 60 mM sodium acetate and 20 mM Na_2EDTA, pH 7) and dimethylsulfoxide (DMSO, Fluka, analytical grade, 41640) in a ratio of 50:8:100 was prepared, 16 µl of this was added to 20 µg of RNA (Section 2.18) in a 4 µl volume (under light mineral oil) and this was incubated at 50°C for 1 h.

2.19.2 Glyoxal Gel

A surface tension 1% agarose gel in 1 x MOPS buffer (Section 2.19.1) was poured on a pre-warmed 12.5 x 10 cm glass plate. All 20 µl of the glyoxylation reaction (prepared as in Section 2.19.1) was loaded, and SDS loading dye was loaded in an adjacent lane. The gel was run at 70 V in a cold room (4°C) until the marker dye had

migrated 4/5 the length of the gel. The gel was then stained with ethidium bromide (0.25 μg/ml) and the RNA visualised on a UV transilluminator then photographed.

2.19.3 Northern Blotting and Hybridisation

RNA from the glyoxal gel (Section 2.19.2) was transferred to a Nylon membrane (Amersham, Hybond N) by capillary blotting as in Section 2.12.1 except the gel was not pre-treated in any way and the transfer was performed overnight at 4°C. The unwashed membrane was baked at 80°C for 2 h in a vacuum oven to reverse glyoxylation and fix RNA onto the membrane. Preparation of [α-^{32}P]dCTP labelled probe DNA, hybridisation and autoradiography was performed as in Section 2.12.2-2.12.5.

2.20 RT-PCR ANALYSIS OF RNA

This method was based on that of Kawasaki (1990). The precautions given in Section 2.17 were taken throughout.

2.20.1 Reverse Transcriptase Synthesis of cDNA

Before cDNA synthesis and PCR amplification of cDNA it was first necessary to remove all traces of contaminating DNA from the RNA preparation. RNA (10 μg, Section 2.18) was treated with 20 units of DNaseI (Boehringer) in the presence of 5 μl of pancreatic RNase inhibitor (BRL) and 1 μl of 100 mM dithiothreitol in 200 μl of DNase buffer (100 mM sodium acetate, 5 mM magnesium sulphate, pH 5). This was incubated at 37°C for 30 min, another 20 units of DNaseI then added and the reaction incubated at 37°C for another 30 min. This was followed by a phenol/chloroform extraction and the RNA was resuspended in 20 μl of TE (10/1).

In a microcentrifuge tube 2.0 μl of 10 x RT-PCR buffer (500 mM KCl, 200 mM Tris-HCl [pH 8.4 at 23°C], 25 mM $MgCl_2$, and 1 mg/ml bovine serum albumin), 1 mM of each dNTP, 20 units of human pancreatic RNase inhibitor (BRL), 100 pmol of random primers (BRL), 1 μg of DNaseI treated total RNA (from above, heated to 90°C for 5 min then cooled on ice immediately before adding to the reaction), 200 units of Superscript reverse transcriptase (BRL) and MilliQ water to 20 μl were mixed and incubated at room temperature for 10 min, then at 42°C for 45 min. The reaction was then stored at -20°C.

2.20.2 Amplification of cDNA by PCR

To 10 μl of the cDNA reaction from Section 2.20.1, 10 pmol of each of the two primers for amplification of the target sequence of interest, 4 μl of 10 x RT-PCR

buffer, 2.5 units of Taq DNA polymerase (Boehringer) and MilliQ water to 50 µl was added. The reaction vessels were placed in the thermal cycler preheated to 94°C and after an initial 2 min melt at 94°C subjected to 35 cycles of 94°C for 45 sec, 58°C for 45 sec and 72°C for 60 sec. After the 35 cycles were complete the reactions were incubated at 72°C for 5 min, then stored at 4°C or -20°C.

2.21 PREPARATION OF FUNGAL PROTOPLASTS

Fungal mycelia (grown as outlined in Section 2.2), was removed from the growth media, either by peeling off cellophane discs, or draining the broth out of the flask and 4 ml of sterile 5 mg/ml Novozyme in OM buffer (Section 2.4.13) was added to each cellophane disc or flask containing fungal mycelia. This was incubated at 30°C with shaking until the solution cleared and numerous protoplasts were visible in the solution when examined under a microscope. The protoplasts were then harvested by overlaying 5 ml of the protoplasts solution with 1 ml of ST buffer (0.6 M Sorbitol, 100 mM Tris-HCl pH 8.0) and centrifuging for 5 min at 3 000 g. The protoplasts formed a clear white layer between the Novozyme solution and ST buffer, these were removed and resuspended in 5 ml of STC (Section 2.4.14), pelleted by centrifugation at 7 700 g and then resuspended in 0.5 ml of STC. A sample of protoplasts was diluted in STC buffer, the concentration of the protoplasts estimated by counting on a haemocytometer, and the stock diluted with STC buffer to 1.25×10^8/ml. This method was based on the method of Murray *et al.* (1992).

2.22 TRANSFORMATION OF *ASPERGILLUS NIDULANS*

In a microcentrifuge tube 20 µl of 40% PEG solution (40% [w/v] PEG 4000, 50 mM $CaCl_2$, 50 mM Tris-HCl, pH 8.0, and 1 M sorbitol), 2 µl of 50 mM spermidine, 5 µl of heparin (5 mg/ml in STC buffer, Section 2.4.14) and 5 µg of DNA (1 µg/ml) was added to 80 µl of protoplasts (prepared as in Section 2.21), vortexed briefly then incubated on ice for 30 min, 900 µl of 40% PEG solution was then added, vortexed briefly then incubated at room temperature for 15 min. This solution was then diluted 10 x in STC buffer and 100 µl aliquots plated in 5 ml of molten ACM+S (Section 2.3.2) containing 0.8% agar onto ACM+S plates supplemented as required. Plates were incubated at 37°C and scored for transformants 2-4 days later. Transformants were subcultured onto suitable selective media. A negative control (no DNA) was always included. This method was based on the method of Murray *et al.* (1992).

2.23 SEPARATION OF FUNGAL CHROMOSOMAL DNA BY PULSE FIELD GEL ELECTROPHORESIS

2.23.1 Preparation of Fungal DNA for Pulse Field Gel Electrophoresis

Protoplasts were prepared as in Section 2.21 except they were resuspended in 0.5 ml of GMB buffer (125 mM Na_2EDTA, 900 mM sorbitol, pH 7.5) instead of STC. The protoplast solution was heated to 45°C and an equal volume of molten 1.2% low melting point (LMP) agarose (in GMB buffer) was added, the solution vortexed, pipetted into plug moulds and set at 4°C. The plugs were incubated at 55°C in 10 ml of SE buffer (2% [w/v] sodium dodecyl sulphate, 250 mM Na_2EDTA, pH 8.0) for 16-20 h and then transferred to 10 ml of 10 x ET buffer (500 mM Na_2EDTA, 10 mM Tris-HCl, pH 8) containing 1% (w/v) sodium lauroyl sarcosine and 20 mg of proteinase K and incubated at 50°C for 24 h. The plugs were then washed twice in 1 x ET buffer at 4°C (10 x dilution of 10 x ET buffer) over a period of several hours and stored at 4°C in 1 x ET buffer. This method was based on that of Murray *et al.* (1992).

2.23.2 Pulse Field Gel Electrophoresis

The plugs (Section 2.23.1) were loaded onto a 0.6% (w/v) chromosomal grade agarose gel, sealed with 0.6% LMP and the chromosomal DNA separated by contour clamped homogenous electric field gel electrophoresis using a CHEF DRII (Biorad) electrophoresis unit at 14°C in 0.5 x TBE run at 60 V with pulse intervals of 2 min, 7.5 min, 26.5 min and 33.5 min for total times of 15 h, 13 h, 35 h and 35 h respectively. Gels were stained in ethidium bromide (1 µg/ml) for 30 min, then destained in MilliQ water for 30 min before photography. This method was based on that of Murray *et al.* (1992).

Chapter 3.0 RESULTS

3.1 DETERMINING CONDITIONS FOR LIBRARY SCREENING

3.1.1 Determining Hybridisation and Washing Temperature for Library Screening

A 0.65 kb pRS4 (see Appendix 2 for map) *Stu*I fragment, containing two conserved domains (P. Tudzynski, pers. comm.), from *Claviceps purpurea pyr*4 (Table 1) was chosen as a suitable probe for attempting to detect Lp1 *pyr*4 . Southern blots containing digests (Section 2.9) of total DNA from Lp1 and pFB6 (Table 1, see Appendix 2 for map) were hybridised to [α-^{32}P]dCTP-labelled 0.65 kb pRS4 *Stu*I fragment (Section 2.12) in 3 x SSC at 55°C, 60°C and 65°C then washed at the same temperatures in 3 x SSC, 0.1% SDS (three washes of 30 minutes each) and 1 x SSC, 0.1% SDS (one wash for 30 minutes) and autoradiographed overnight. Hybridisations and washes at 65°C gave the clearest signal (Fig. 5), so all subsequent hybridisations were at this temperature.

3.1.2 Determining Post Hybridisation Washing Conditions for Library Screening

To further define the optimal stringency for library screening, a Southern blot containing digests (Section 2.9) of Lp1 total DNA and pFB6 was hybridised to [α-^{32}P]dCTP-labelled 0.65 kb pRS4 *Stu*I fragment (Section 2.12) at 65°C then washed at the same temperature, initially in 3 x SSC, 0.1% SDS, followed by 1 x SSC, 0.1% SDS, then 0.5 x SSC, 0.1% SDS and finally 0.25 x SSC, 0.1% SDS (each wash for 30 minutes). The blot was autoradiographed wet between washes. Washes in 1 x SSC, 0.1% SDS for 30 minutes gave the strongest signal with the least background (Fig. 6), so these conditions were chosen for screening the library with the 0.65 kb pRS4 *Stu*I fragment and all subsequent washes were performed at this stringency.

3.2 LIBRARY SCREENING AND MAPPING POSITIVE CLONES

3.2.1 Library Screening

Filters containing approximately 45,000 pfu from a λ EMBL3A genomic library prepared from Lp1 and named L5/6 (DNA prepared by F. R. Murray, library prepared by C. A. Young and D. B. Scott, see Appendix 1 for details on construction) were screened by plaque hybridisation (Section 2.13) with [α-^{32}P]dCTP-labelled 0.65 kb pRS4 *Stu*I fragment (Section 2.12.2) using conditions determined in Section 3.1.1 (ie. 3 x SSC at 65°C). Filters were washed using conditions determined in Section 3.1.2

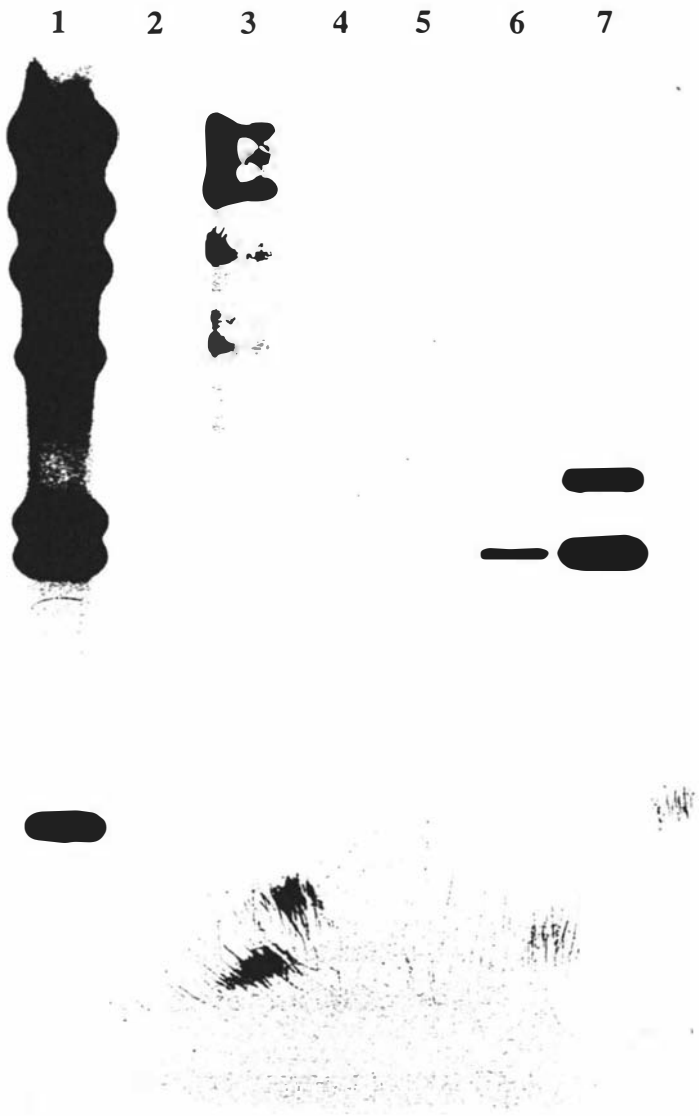


Fig. 5. Establishing optimal temperature for hybridisation of *Claviceps purpurea pyr*4 to Lp1 total DNA.

Hybridisation of 0.65 kb pRS4 *Stu*I fragment to total DNA from Lp1 and *Neurospora crassa pyr*4. Autoradiographs of Southern blots of 5 µg *Eco*RI digested Lp1 total DNA (lane 3) and 85 pg (lane 5), 850 pg (lane 6) and 8.5 ng (lane 7) *Pvu*II and *Bgl*II digested pFB6 DNA hybridised to [α-^{32}P]dCTP-labelled 0.65 kb pRS4 *Stu*I fragment, then washed in 3 x SSC, 0.1% SDS and 1 x SSC, 0.1% SDS. Hybridisations and washes were at 65°C. Lane 1 contains *Hin*dIII digested λ DNA labelled with [α-^{32}P]dCTP.

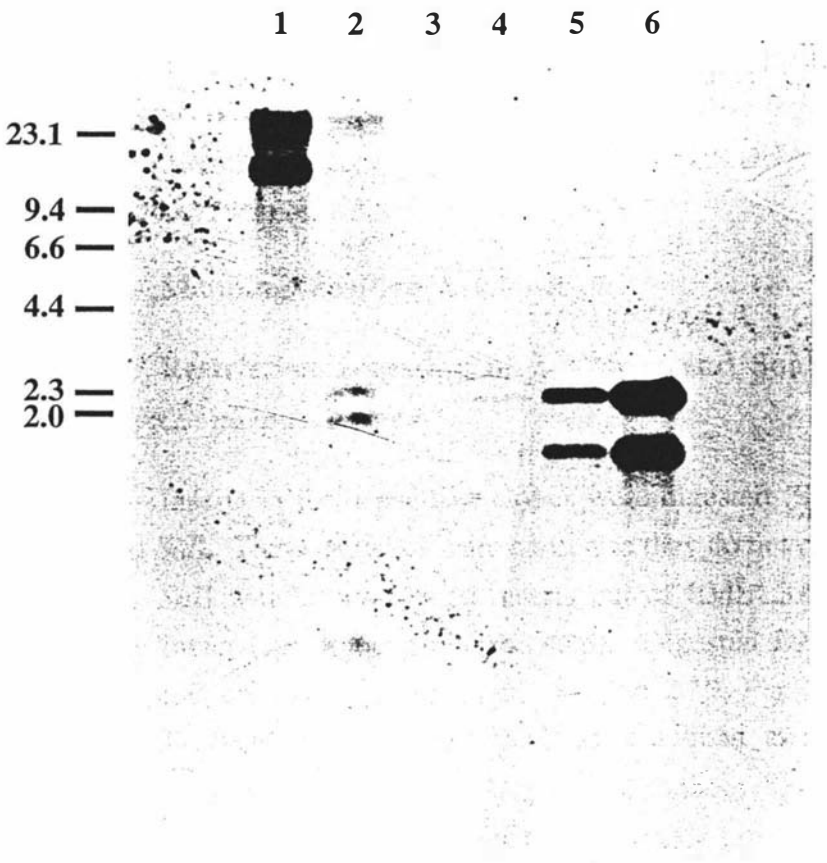


Fig. 6. Establishing salt concentration for post-hybridisation washing of *Claviceps purpurea pyr*4 off Lp1 total DNA.

Hybridisation of 0.65 kb pRS4 *Stu*I fragment to total DNA from Lp1 and *Neurospora crassa pyr*4. Autoradiograph of a Southern blot of 5 µg *Eco*RI digested Lp1 total DNA (lane 1), 5 µg *Hin*dIII digested Lp1 total DNA (lane 2) and 85 pg (lane 4), 850 pg (lane 5) and 8.5 ng (lane 6) *Pvu*II and *Bgl*II digested pFB6 DNA hybridised to [α-^{32}P]dCTP labelled 0.65 kb pRS4 *Stu*I fragment at 65°C, then washed in 3 x SSC, 0.1% SDS then 1 x SSC, 0.1% SDS. Numbers on the left of the figure indicate the position and size (kb) of fragments generated by a *Hin*dIII digest of λ DNA.

(ie. at 65°C, first in 3 x SSC, 0.1% SDS and finally in 1 x SSC, 0.1% SDS for 30 minutes each). Initially 8 positive plaques were obtained, however on the second screen only 4 (λMC11, λMC12, λMC14 and λMC20) of these 8 plaques remained positive. After another round of plaque purification (Section 2.13) DNA was isolated from the four positive clones (Section 2.5.8).

3.2.2 Mapping Positive λ Clones

3.2.2.1 Restriction Digestion of λ Clones and Southern Hybridisation to *C. purpurea pyr*4

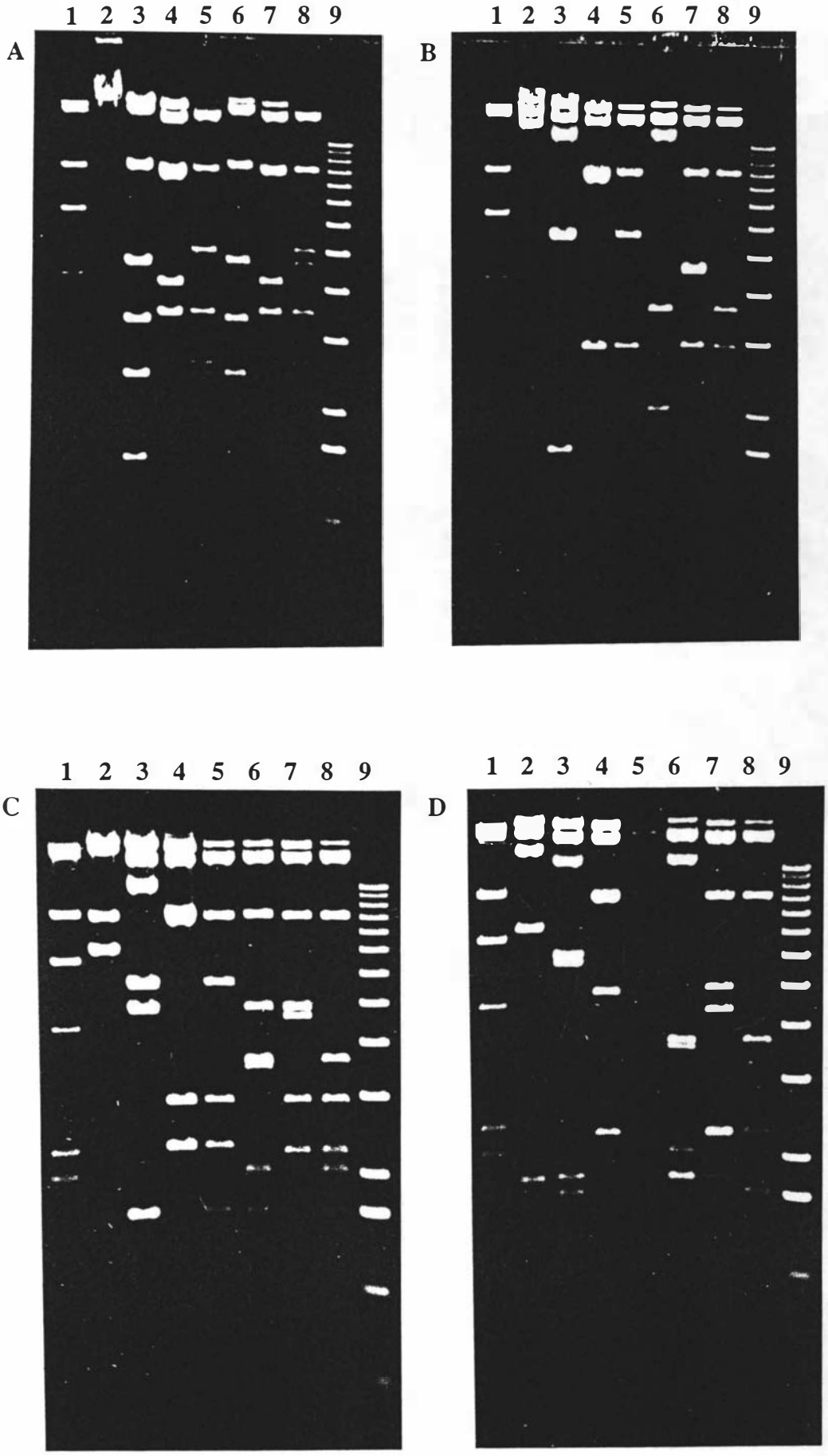
Inserts in the 4 positive clones were digested (Section 2.9) with *Bam*HI, *Eco*RI and *Sal*I. These enzymes were chosen as they do not cut within EMBL3A arms, except for *Sal*I which cuts cloned inserts out of EMBL3A arms, leaving the arms essentially intact (see Appendix 2 for map). Digested DNA was separated by gel electrophoresis on a 0.7% agarose gel (Section 2.10, Fig. 7A-F). Triple digests of λMC11 with *Bam*HI, *Eco*RI and *Sal*I gave a band at about 4.7 kb which was inconsistent with other digests (Fig. 7A, lane 8), so these digests were repeated and this 4.7 kb band found to be an artefact (Fig. 7E), probably a result of restriction enzyme star activity. The *Bam*HI, *Sal*I double digest of λMC20 gave degraded DNA (Fig. 7D, lane 5) so was repeated (Fig. 7F). Information from these digests is summarised in Table 2. Clones λMC12, λMC14 and λMC20 shared a number of common bands within their inserts, but λMC11 shared no common genomic restriction fragments with these clones. Southern blots of these gels were hybridised to an [α-^{32}P]dCTP-labelled (Section 2.12) 0.65 kb pRS4 *Stu*I fragment. These blots revealed that all four λ clones contained fragments hybridising to this *pyr*4 probe (fragments that hybridised are identified by an asterisk in Table 2), however fragments from λMC11 which hybridised differed in size to those from the other three λ clones (Fig. 8A-F, Table 2) suggesting it was from a different genetic region.

3.2.2.2 Mapping λ Clones with *Bam*HI and *Sal*I Using a λ Mapping System

The complexity of fragments from restriction digests (summarised in Table 2) made mapping of these clones difficult, so a λ mapping system was employed. This involved partial digestion of λ clones with *Bam*HI or *Sal*I, hybridisation of either of two 5' end-labelled oligonucleotide (ON-L or ON-R) to partially digested DNA and separation of partially digested fragments on a 0.4% agarose gel which was then dried and autoradiographed (Section 2.14 and Fig. 9). Oligonucleotides ON-L and ON-R are

Fig. 7A-F. Restriction digestion profiles of λMC11, λMC12, λMC14 and λMC20.

*Eco*RI (lane 2), *Bam*HI (lane 3), *Sal*I (lane 4), *Bam*HI and *Sal*I (lane 5), *Bam*HI and *Eco*RI (lane 6), *Eco*RI and *Sal*I (lane 7) and *Bam*HI, *Eco*RI and *Sal*I (lane 8) digests of: **A** λMC11; **B** λMC12; **C** λMC14; **D** λMC20; **E** λMC11; and **F** λMC20 flanked by *Hind*III digested λ DNA (lane 1) and 1 kb BRL ladder (lane 9).


1 2 3 4 5 6 7 8 9
A
1 2 3 4 5 6 7 8 9
B
1 2 3 4 5 6 7 8 9
C
1 2 3 4 5 6 7 8 9
D

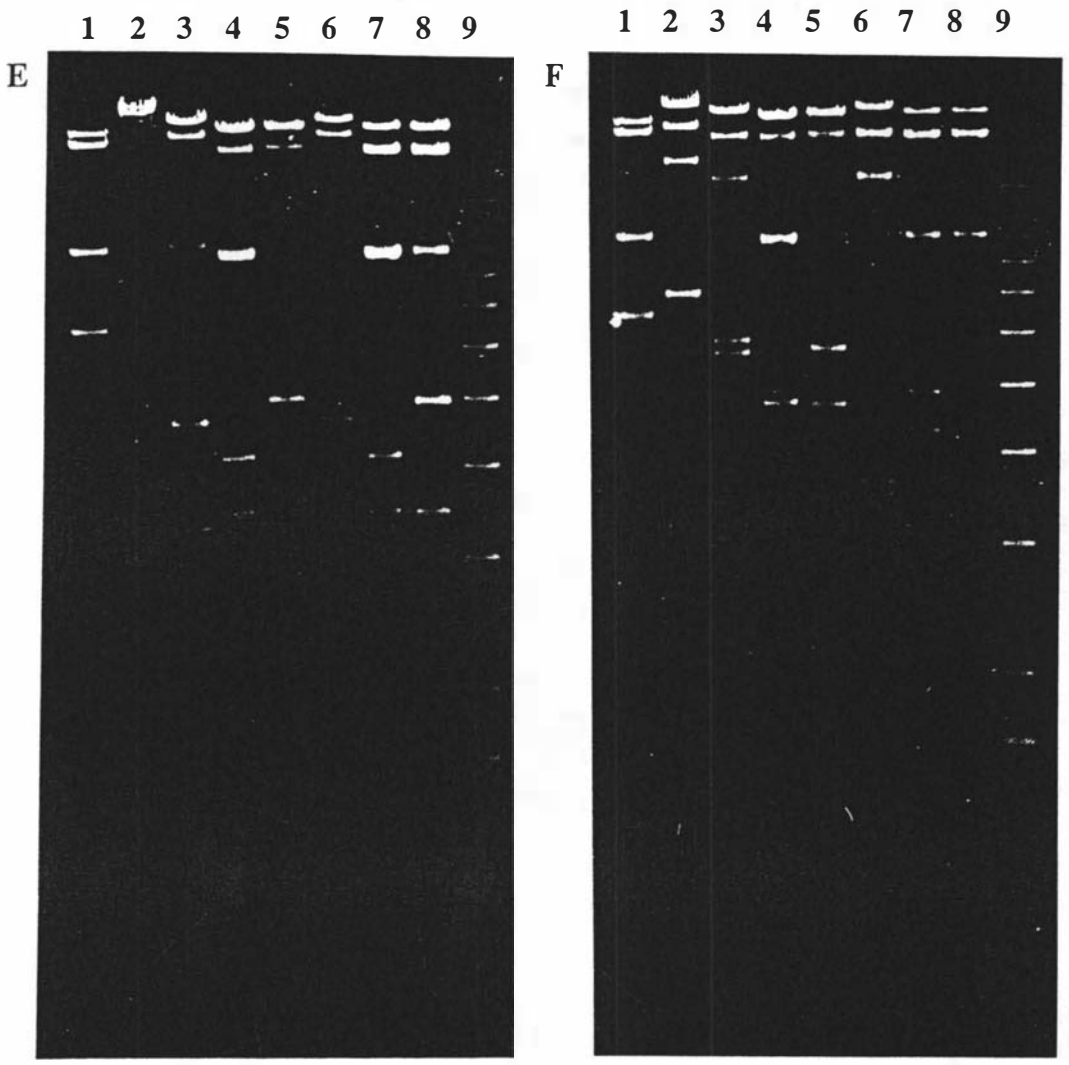

1 2 3 4 5 6 7 8 9
E
1 2 3 4 5 6 7 8 9
F

Table 2 Data from restriction mapping of λ clones from Fig. 7 and Fig. 8.

λ Clone	Restriction digest	Fragment Size (kb)
λMC11	*Eco*RI	>27.5*
	*Bam*HI	25.5, 9.4, 4.5, 3.4*, 2.4*, 1.4, 0.3
	*Sal*I	19.9, 8.8, 8.5*, 4.4*, 3.4, 0.96
	*Bam*HI / *Sal*I	19.9, 8.8, 5.1, 3.4, 2.6*, 2.4*, 1.4, 0.9, 0.8*, 0.7, 0.3
	*Bam*HI /*Eco*RI	25.5, 9.4, 4.5, 3.4*, 2.4*, 1.4, 0.3
	*Sal*I / *Eco*RI	19.9, 8.8, 8.5*, 4.4*, 3.4, 0.96
	*Bam*HI / *Eco*RI / *Sal*I	19.9, 8.8, 5.1, 3.4, 2.6*, 2.4*, 1.4, 0.9, 0.8*, 0.7, 0.3
λMC12	*Eco*RI	25, 18*
	*Bam*HI	21, 14.5, 5.8*, 1.6*
	*Sal*I	19.9, 9.4*, 8.8, 3.0, 1.2, 0.5
	*Bam*HI / *Sal*I	19.9, 8.8, 5.8*, 3.0, 1.6*, 1.2, 1.1, 1.05, 0.5
	*Bam*HI /*Eco*RI	21, 14.5, 3.6, 2.2*, 1.6*
	*Sal*I / *Eco*RI	19.9, 8.8, 4.8*, 4.6, 3.0, 1.2, 0.5
	*Bam*HI / *Eco*RI / *Sal*I	19.9, 8.8, 3.6, 3.0, 2.2*, 1.6*, 1.2, 1.1, 1.05, 0.5

Table 2 Continued

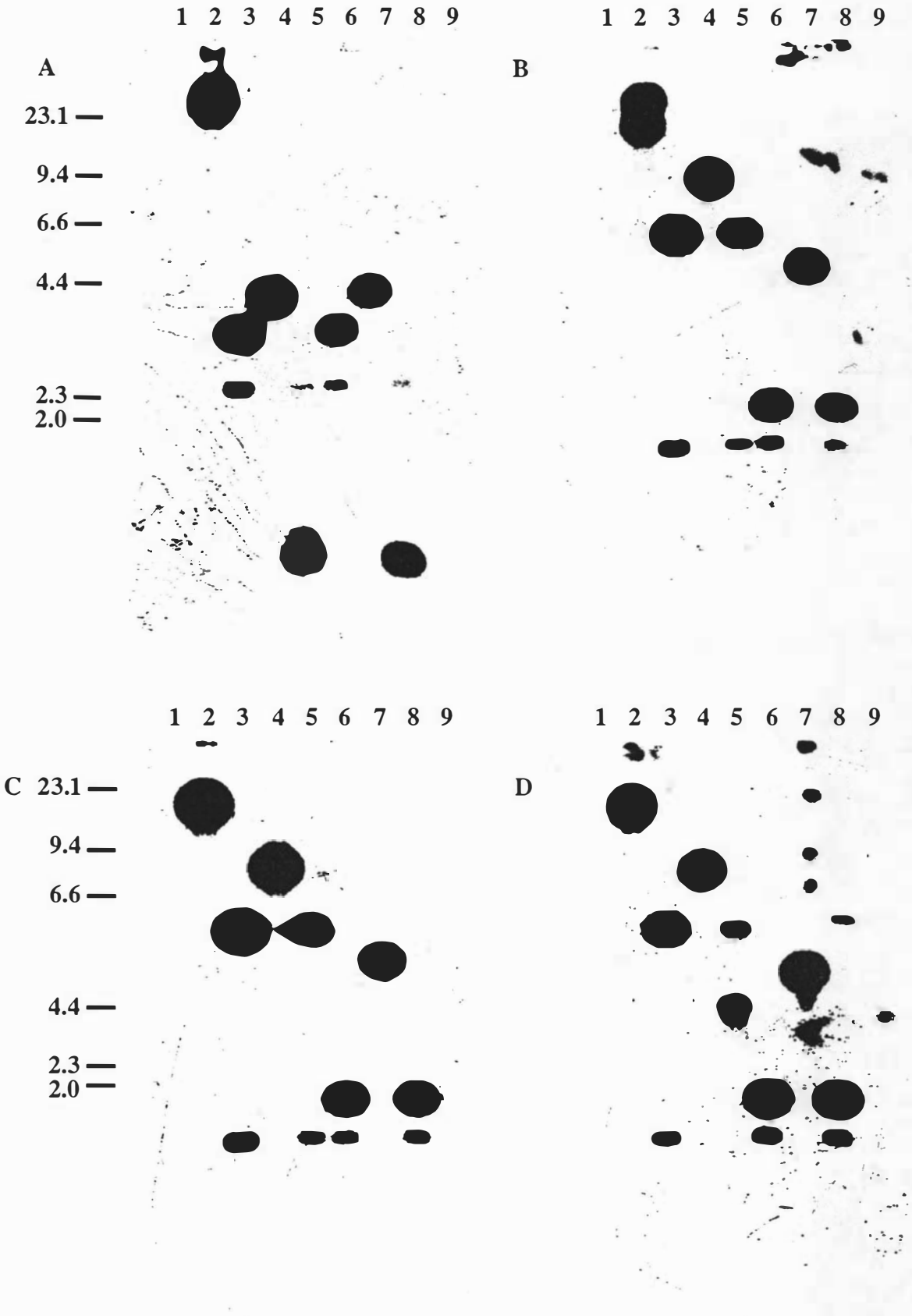
λ Clone	Restriction digest	Fragment Size (kb)
λMC14	*Eco*RI	>27.5*, 9.4, 7.2
	*Bam*HI	20, 12.5, 5.8*, 4.8[a], 1.6*
	*Sal*I	19.9, 9.7*, 8.8, 3.0, 2.3, 0.5
	*Bam*HI / *Sal*I	19.9, 8.8, 5.8*, 3.0, 2.3, 1.6*, 1.3, 1.0, 0.5
	*Bam*HI /*Eco*RI	20, 9.4, 4.8, 3.6, 3.4, 2.2*, 1.6*
	*Sal*I / *Eco*RI	19.9, 8.8, 4.9, 4.8*, 3.0, 2.3, 0.5
	*Bam*HI / *Eco*RI / *Sal*I	19.9, 8.8, 3.6, 3.0, 2.2*, 2.0, 1.6*, 1.3, 1.0, 0.5
λMC20	*Eco*RI	25, 16, 7.2, 1.7
	*Bam*HI	20, 13, 6.0, 5.8*, 1.7, 1.6*
	*Sal*I	19.9, 9.4*, 8.8, 4.7, 2.3, 1.2, 0.8, 0.8, 0.5, 0.2
	*Bam*HI / *Sal*I	
	*Bam*HI /*Eco*RI	20, 13, 3.6, 3.6, 2.2*, 1.7, 1.7, 1.6*, 0.7
	*Sal*I / *Eco*RI	19.9, 8.8, 4.9, 4.5*, 2.3, 2.2, 1.7, 1.2, 0.8, 0.8, 0.7, 0.5, 0.2
	*Bam*HI / *Eco*RI / *Sal*I	19.9, 8.8, 3.6, 2.3, 2.2*, 1.7, 1.7, 1.6*, 1.2, 1.2, 0.8, 0.8, 0.7, 0.7, 0.6, 0.5, 0.2

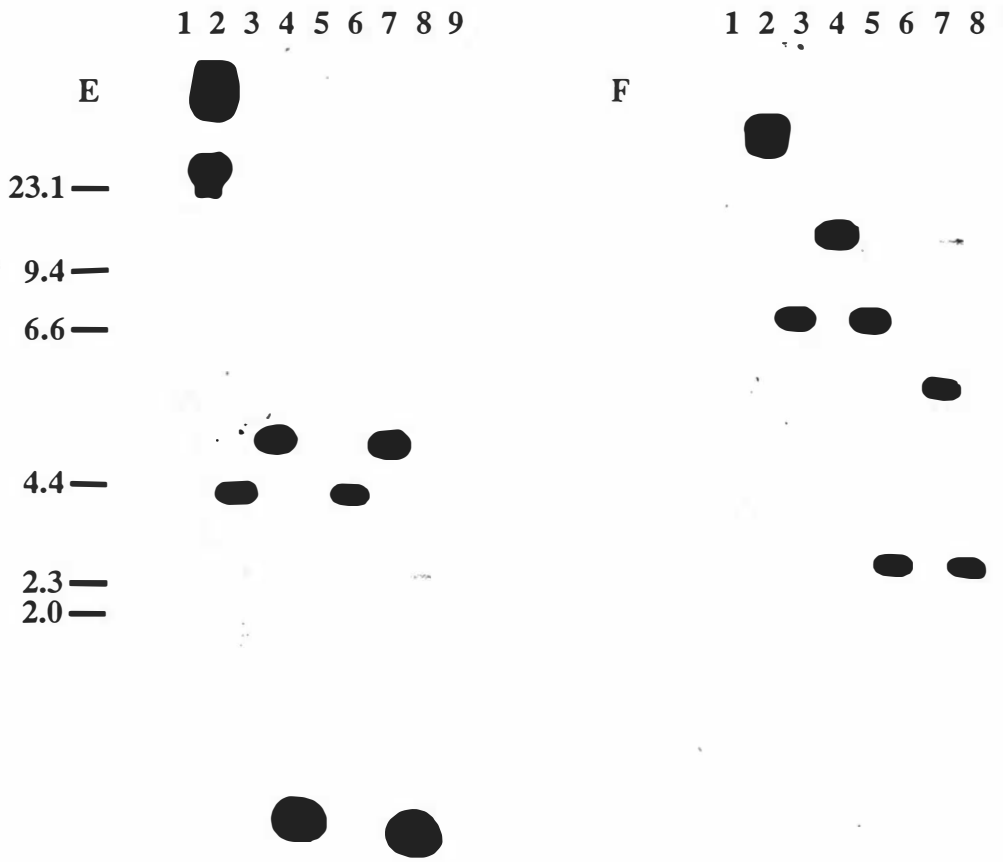
* Indicates fragments in Fig. 8 hybridising to [α-^{32}P] labelled 0.65 kb pRS4 *Stu*I fragment.

[a] This band presumably created by ligation of *Mbo*I partially digested fragment containing half a *Bam*HI site into *Bam*HI cut λ EMBL3A arm.

Fig. 8A-F. Mapping the position of *pyr*4 on λMC11, λMC12, λMC14 and λMC20.

Autoradiograph of Southern blots of gels shown in Fig. 7 hybridised to [α-^{32}P]dCTP labelled 0.65 kb pRS4 StuI fragment. *Eco*RI (lane 2), *Bam*HI (lane 3), *Sal*I (lane 4), *Bam*HI and *Sal*I (lane 5), *Bam*HI and *Eco*RI (lane 6), *Eco*RI and *Sal*I (lane 7) and *Bam*HI, *Eco*RI and *Sal*I (lane 8) digests of: **A** λMC11; **B** λMC12; **C** λMC14; **D** λMC20; **E** λMC11; and **F** λMC20 flanked by *Hind*III digested λ DNA (lane 1) and the 1 kb BRL ladder (lane 9).


1 2 3 4 5 6 7 8 9
A
23.1
9.4
6.6
4.4
2.3
2.0
B
C
D


1 2 3 4 5 6 7 8 9
E
23.1
9.4
6.6
4.4
2.3
2.0
1 2 3 4 5 6 7 8 9
F

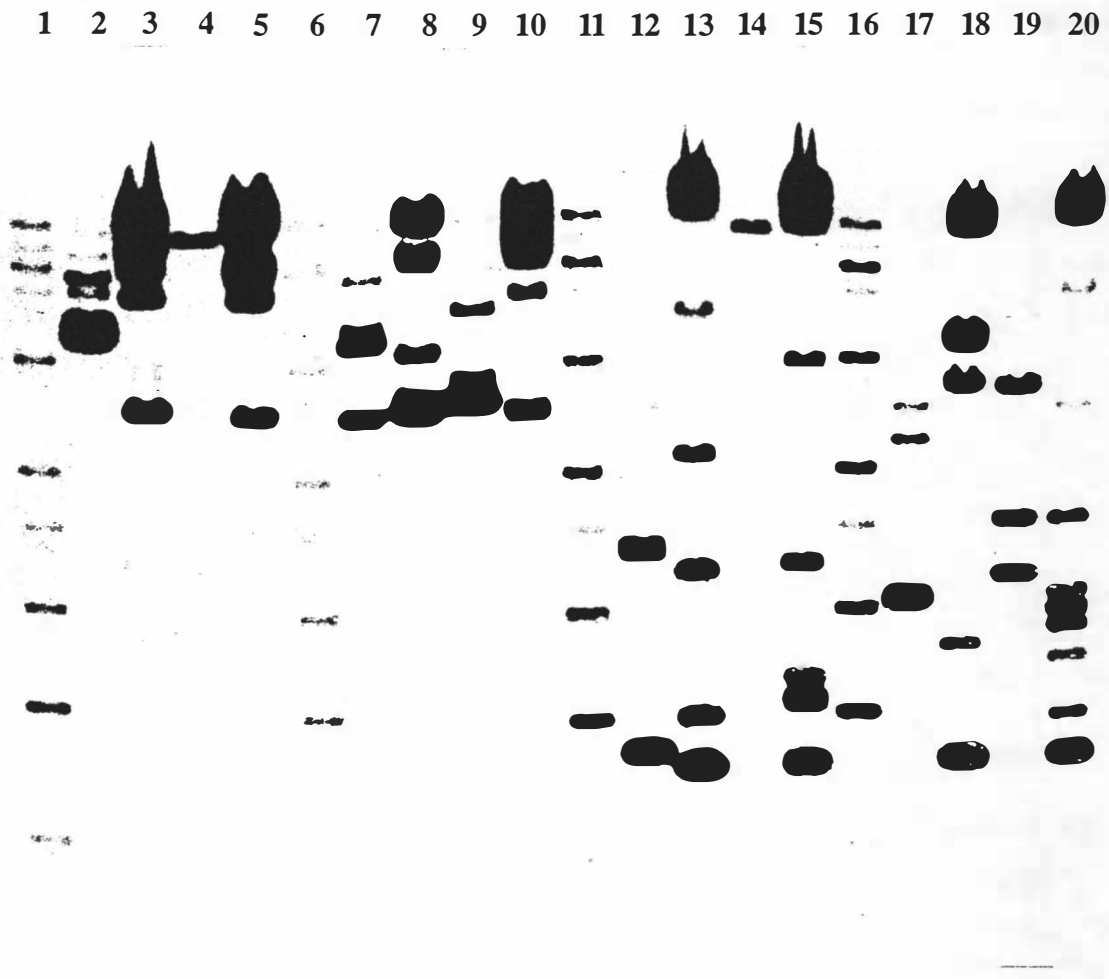


Fig. 9. Profiles of λMC11, λMC12, λMC14 and λMC20 partially digested with *Bam*HI or *Sal*I and hybridised to ON-L or ON-R.

Partial digests of λ clones: λMC11 digested with *Bam*HI (lane 2), λMC11 digested with *Sal*I (lane 3), λMC12 digested with *Bam*HI (lane 4), λMC12 digested with *Sal*I (lane 5), λMC14 digested with *Bam*HI (lane 7), λMC14 digested with *Sal*I (lane 8), λMC20 digested with *Bam*HI (lane 9), λMC20 digested with *Sal*I (lane 10), λMC11 digested with *Bam*HI (lane 12), λMC11 digested with *Sal*I (lane 13), λMC12 digested with *Bam*HI (lane 14), λMC12 digested with *Sal*I (lane 15), λMC14 digested with *Bam*HI (lane 17), λMC14 digested with *Sal*I (lane 18), λMC20 digested with *Bam*HI (lane 19), λMC20 digested with *Sal*I (lane 20). Partially digested λ clones in lanes 2-10 were hybridised to [γ-^{32}P] 5' end-labelled ON-L and those in lanes 12-20 were hybridised to [γ-^{32}P]-end labelled ON-R. Lanes 1, 6, 11 and 16 contained λ size fragment markers (48.5, 38.4, 33.5, 29.9, 23.1, 17.1, 15.0, 12.4, 10.1 and 7.9 kb) hybridised to labelled ON-L and ON-R.

complementary to single stranded *cos* ends of EMBL3A left and right arms respectively, allowing detection by autoradiography only of partially digested DNA fragments which have not been cleaved from these arms. This allows the order and distance between all restriction sites in the λ clones to be calculated, as descending bands on the autoradiograph correspond to various partial restriction fragments which still have an intact arm at one (or both) ends. The order of restriction fragments of the λ clones determined in this way is summarised in Table 3. This information, in addition to information in Table 2, was used to construct *Bam*HI and *Sal*I restriction maps of the four λ clones (Fig. 10).

During λ mapping of clone MC12 the *Bam*HI partial restriction digest failed to cut this clone for unknown reasons (lanes 4 and 14, Fig. 9 and Table 3), despite the presence of *Bam*HI sites in λMC12 (demonstrated in Fig. 7). As *Bam*HI fragments from λMC12 and λMC14 are similar (Table 2) the λMC14 *Bam*HI map was used to construct the *Bam*HI map for λMC12. A 0.5 kb λMC14 *Sal*I fragment, identified in restriction digests (Table 2), was not identified by λ mapping of λMC14 with *Sal*I (Table 3). As a 0.5 kb *Sal*I fragment was present in λMC12 in a region where λMC12 and λMC14 overlap, and no 0.5 kb *Sal*I fragment was placed in this position by λ mapping, the 0.5 kb *Sal*I fragment was placed in this position on the λMC14 map. Examination of λ mapping data in Fig. 9 indicates that bands immediately adjacent to where this fragment was placed are probably doublets, confirming placement of this 0.5 kb *Sal*I fragment at this position.

3.2.2.3 Mapping λ Clones Further

To combine *Bam*HI and *Sal*I maps they were overlaid, using positions of left and right arms to orientate the clones. Data from the double digests with *Bam*HI and *Sal*I (Table 2) was used to further refine the maps, ensuring alignment of the two maps was accurate. Information from the *Eco*RI digest, double digest with *Bam*HI and *Eco*RI, double digests with SalI and *Eco*RI and triple digests with *Bam*HI, *Eco*RI and *Sal*I (Table 2) was used to map *Eco*RI sites of the λ clones. λMC11 contained no *Eco*RI sites and λMC12 contained one *Eco*RI site. The 5.8 kb *Bam*HI fragment of λMC12 was digested by *Eco*RI to give 2.2 kb and 3.6 kb *Bam*HI-*Eco*RI fragments, while the 9.4 kb *Sal*I fragment was digested by *Eco*RI to give 4.8 kb and 4.6 kb *Sal*I-*Eco*RI fragments. Using hybridisation information of *C. purpurea pyr*4 to these and other fragments (Table 2) it was possible to position the *Eco*RI site within the 5.8 kb *Bam*HI and 9.4 kb *Sal*I fragments. To map *Eco*RI sites in other λ clones, maps of λMC12, 14 and 20 were overlaid, and the *Eco*RI site in λMC12 was then used to map other *Eco*RI fragments in λMC14 and 20. In this way complete maps for all four λ clones were obtained for

Table 3 Data from λ mapping of clones

Restriction Enzyme used for Partial Digest	λ Clone (Oligo hybridised to λ DNA)	Fragment Sizes (kb)[a]
*Bam*HI	λMC11 (ON-L)	9, 3.5[b], 3.5, 3.5, 1, 24.5
	λMC11 (ON-R)	25.5, 1, 3, 2.2[c], 0.4[c], 5, 9.4
	λMC12 (ON-L)	42[d]
	λMC12 (ON-R)	46[d]
	λMC14 (ON-L)	12, 5, 2.5, 4.5[e], 20
	λMC14 (ON-R)	18, 5[e], 1.5, 6.2, 12.3
	λMC20(ON-L)	12, 1.5, 5, 5.7, 1.6, 20.2
	λMC20 (ON-R)	20, 2, 6, 5.9, 1.7, 13.5
*Sal*I	λMC11 (ON-L)	10, 5.5[b], 4, 9, 20
	λMC11 (ON-R)	21, 9, 4.6, 3.3[c], 1.2[c], 10
	λMC12 (ON-L)	12[b], 4, 9.5, 20
	λMC12 (ON-R)	20, 9.5, 3, 0.3[c], 1.1[c], 9[c]
	λMC14 (ON-L)	10, 4, 10, 4, 20
	λMC14 (ON-R)	23, 3.5, 10, 2.1, 9
	λMC20 (ON-L)	14[b], 4.4, 10, 20
	λMC20 (ON-R)	19, 9.7, 4.8, 2.3[c], 0.7[c], 0.2[c], 0.9[c], 0.9[c], 1.1[c], 9[c]

[a] Fragment sizes calculated by measuring the size of each band and subtracting the size of the band immediately below.

[b] Two or more bands, unresolved due to high molecular weight of band.

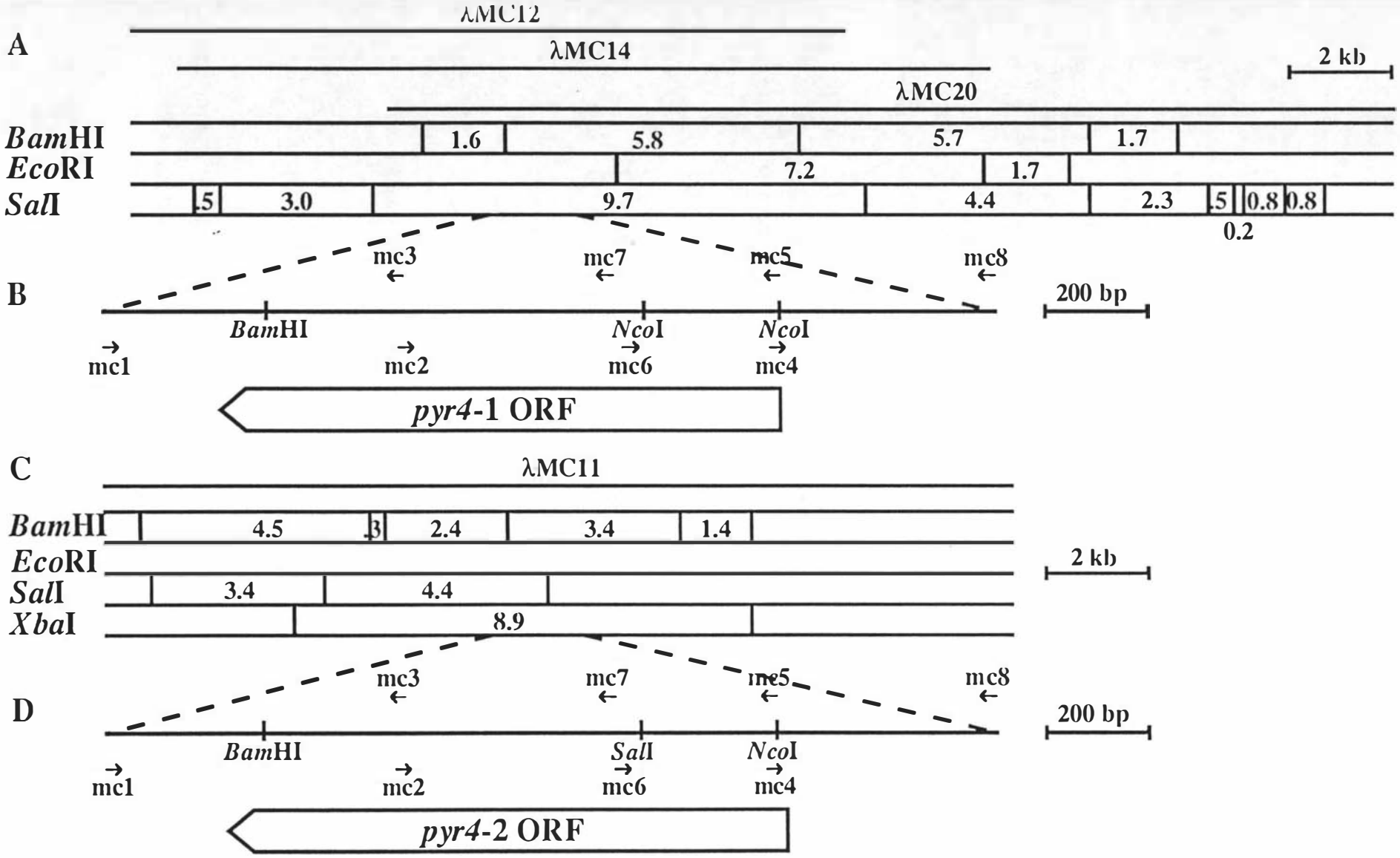
[c] Bands combining to give unresolved bands indicated by [b].

[d] No digestion of λ clone.

[e] This band presumably created by ligation of *Mbo*I partially digested fragment containing half a *Bam*HI site into *Bam*HI cut EMBL3A arm.

Fig. 10. Physical maps of λ clones isolated from an Lp1 genomic library that hybridisaed to *Claviceps purpurea pyr*4.

Restriction maps of genomic regions of two *pyr*4 clones from *Acremonium* strain Lp1. **A, C** Restriction maps of λMC11, λMC12, λMC14 and λMC20 clones. **B, D** Region of these clones sequenced, the *pyr*4-1 and *pyr*4-2 ORFs and primers used for sequencing and PCR.


λMC12
A
λMC14
2 kb
λMC20
BamHI
EcoRI
SalI
1.6
5.8
5.7
1.7
7.2
1.7
.5
3.0
9.7
4.4
2.3
.5
0.8
0.8
0.2
mc3
mc7
mc5
mc8
B
200 bp
BamHI
NcoI
NcoI
mc1
mc2
mc6
mc4
pyr4-1 ORF
C
λMC11
BamHI
EcoRI
SalI
XbaI
4.5
.3
2.4
3.4
1.4
2 kb
3.4
4.4
8.9
mc3
mc7
mc5
mc8
D
200 bp
BamHI
SalI
NcoI
mc1
mc2
mc6
mc4
pyr4-2 ORF

restriction enzymes *Bam*HI, *Eco*RI and *Sal*I (Fig. 10). These maps were consistent with all information in Tables 2 and 3. Details of mapping λMC11 with *Xba*I are given in Section 3.5.1.

This mapping demonstrated that λMC11 was completely independent of the other three clones, and this suggested that two regions had been cloned from Lp1 which were similar to *C. purpurea pyr*4.

3.2.3 Identification of two *pyr*4 Genes in Lp1 Genomic DNA

To demonstrate that two regions hybridising to *C. purpurea pyr*4 identified in the λ clones were present in Lp1 genomic DNA, Southern blots containing total DNA from Lp1, λMC11, λMC12, λMC14 and λMC20 digested (Section 2.9) with *Bam*HI (Fig. 11A) and *Sal*I (Fig. 11B) were hybridised to [α-^{32}P]dCTP-labelled (Section 2.12) pRS4 *Stu*I fragment. This revealed that Lp1 possessed genomic fragments hybridising to *C. purpurea pyr*4 corresponding to fragments of the λ clones.

3.2.4 Confirmation that the λ Clones Isolated Correspond to two Separate Genetic Regions of the Lp1 Genome

To demonstrate that inserts in the four λ clones corresponded to separate genetic regions, and were not cloning artefacts, it was necessary to confirm that restriction fragments of the same size and order as those found in the λ clones were present in Lp1 total DNA. This was achieved by hybridising λMC11 to Southern blots containing *Bam*HI and *Sal*I digests (Section 2.9) of total DNA from Lp1 and the four λ clones (Section 2.12, Figs. 11C and 11D). At a later date a Southern blot containing *Bam*HI digested total DNA from Lp1, the λ clones and other fungal isolates (Fig. 20C) was hybridised to λMC12. These blots demonstrated that genomic *Bam*HI and *Sal*I fragments from λMC11 and λMC12 were present in Lp1 total DNA (Figs. 11C, 11D and 20C). Thus the two different genomic regions which had been cloned were found in Lp1, and were not cloning artefacts. This suggested that either two *pyr*4 genes were present in Lp1 or there was a sequence present which had homology to *C. purpurea pyr*4, but was not an active *pyr*4.

3.3 SEQUENCE ANALYSIS OF CLONED DNA HYBRIDISING TO THE *C. PURPUREA PYR*4 GENE

3.3.1 Subcloning of Regions Required for Sequencing

To further characterise cloned genomic regions, suitable fragments that hybridised to the 0.65 kb pRS4 *Stu*I fragment (Table 2) were subcloned (Section 2.11)

Fig. 11A-B. Demonstration that the inserts present in λ clones correspond to regions of the Lp1 genome.

Hybridisation of 0.65 kb pRS4 *Stu*I fragment to total DNA from Lp1, λMC11, λMC12, λMC14 and λMC20. Autoradiograph of a Southern blot of **A** *Bam*HI digests and **B** *Sal*I digests of total DNA from Lp1 (lane 1), λMC11 (lane 3), λMC12 (lane 4), λMC14 (lane 5) and λMC20 (lane 6) hybridised with [α-^{32}P]dCTP labelled 0.65 kb pRS4 *Stu*I fragment. 5 μg of total DNA from Lp1 and 4.5 ng of DNA from each of the three λ clones was analysed. Numbers on the left of the figure indicate the position and size (kb) of fragments generated by a *Hin*dIII digest of λ DNA.

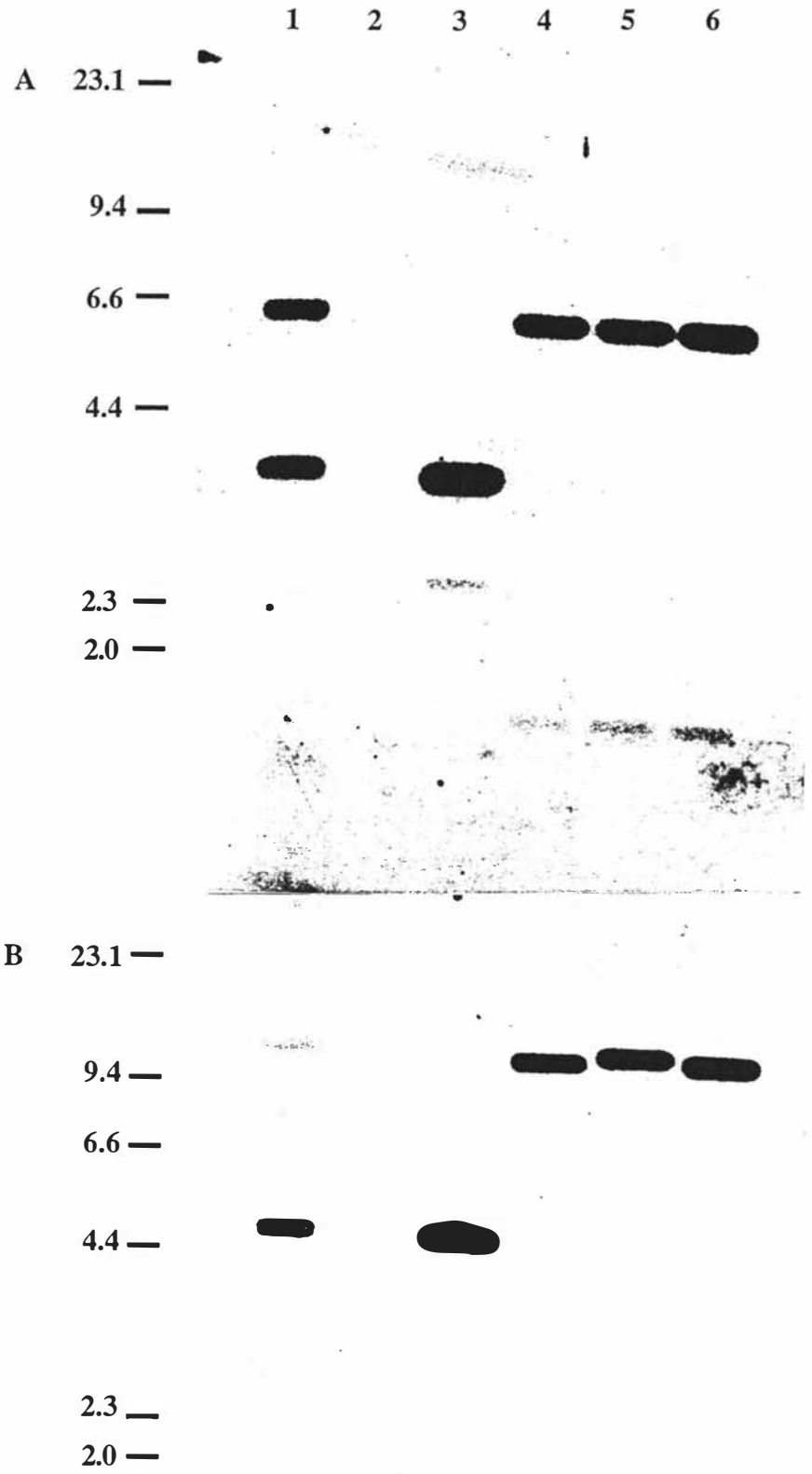
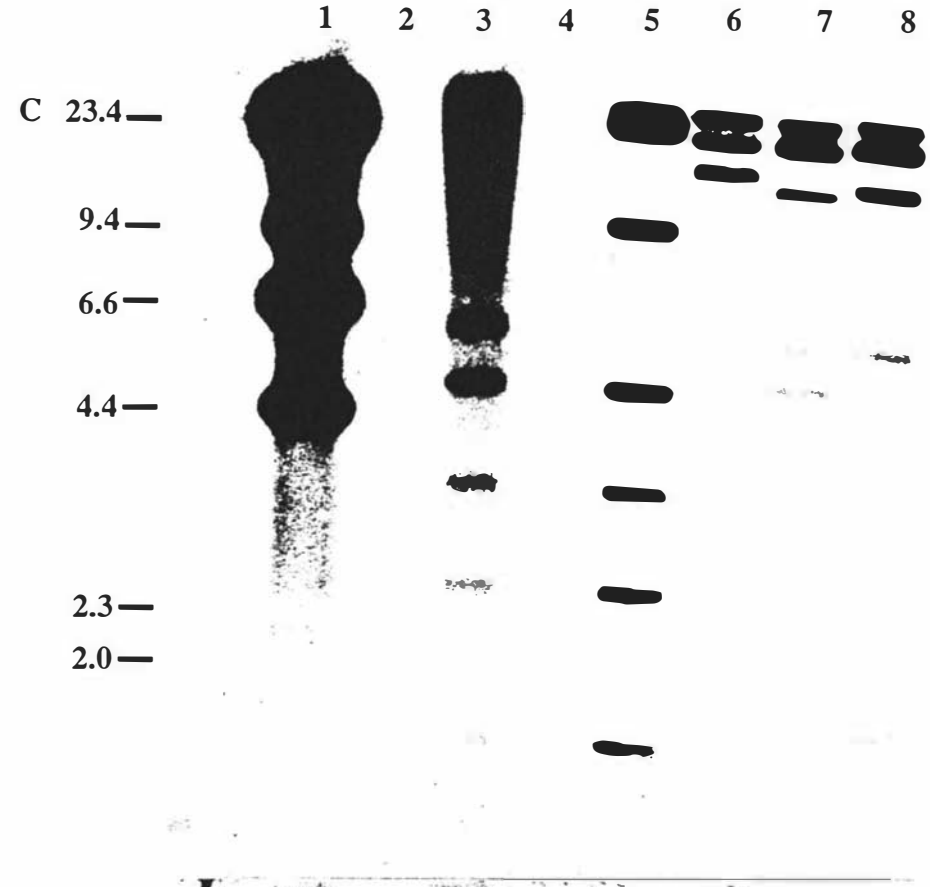

1 2 3 4 5 6
A
23.1
9.4
6.6
4.4
2.3
2.0
B
23.1
9.4
6.6
4.4
2.3
2.0

Fig. 11C-D. Demonstration that the inserts present in λ clones correspond to regions of the Lp1 genome.

Hybridisation of λMC11 to total DNA from Lp1, λMC11, λMC12, λMC14 and λMC20. **C** Autoradiograph of a Southern blot of *Bam*HI digests and **D** *Sal*I digests of total DNA from Lp1 (lane 3), λMC11 (lane 5), λMC12 (lane 6), λMC14 (lane 7) and λMC20 (lane 8) and λ DNA digested with *Hin*dIII (lane 1) hybridised with [α-^{32}P]dCTP labelled λMC11. 5 μg of total DNA from Lp1 and 4.5 ng of DNA from each of the three λ clones was analysed. Numbers on the left of the figure indicate the position and size (kb) of fragments generated by a *Hin*dIII digest of λ DNA.


1
2
3
4
5
6
7
8
C
23.4
9.4
6.6
4.4
2.3
2.0


D
23.1
9.4
6.6
4.4
2.3
2.0

into pUC118 to give plasmids pMC1, pMC2, pMC3, pMC4 and pMC5 (Table 1). The identity of pMC1 was confirmed by hybridisation of the 0.65 kb pRS4 *Stu*I fragment to a Southern blot of pMC1 (Section 2.12, data not shown), so as to distinguish it from another fragment of very similar size in λMC11 which was also subcloned. Identities of other fragments subcloned were confirmed by restriction digestion (Section 2.9) and analysis by gel electrophoresis (Section 2.10, data not shown).

3.3.2 Sequencing and Identification of two *pyr*4 Sequences from Lp1, *pyr*4-1 and *pyr*4-2

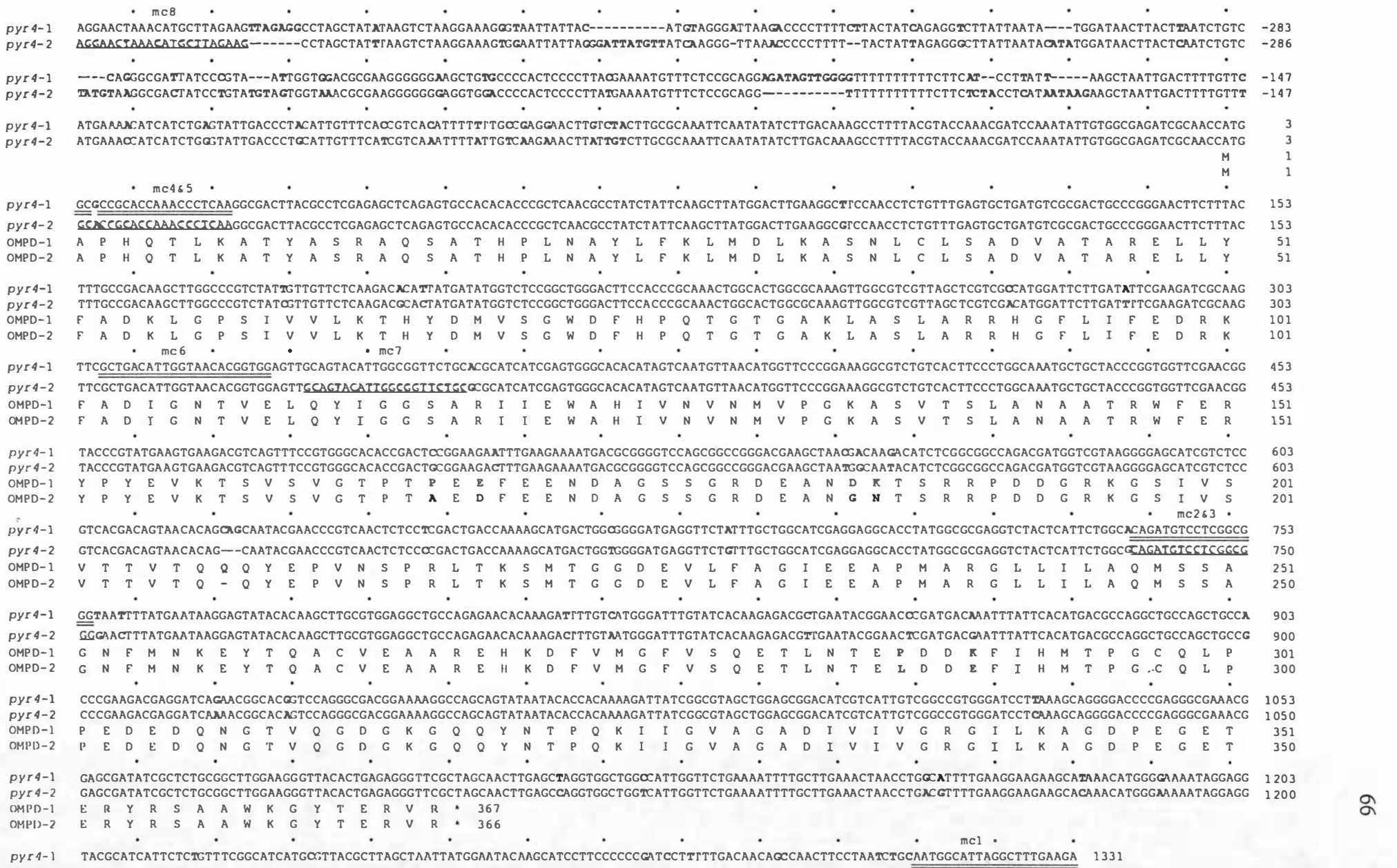
DNA from plasmid subclones (Section 3.3.1) was isolated by a PEG precipitation method (Section 2.5.7), and sequenced using a combination of Sequenase Version 2.0 (Section 2.16.3) and the fmol DNA sequencing system (Section 2.16.2). pUC/M13 "universal" forward (Promega Q560B) and reverse (Promega Q540A) primers were used to obtain insert sequence. This information was used to design primers for further sequencing and PCR. Sequences through restriction sites used for subcloning were confirmed by directly sequencing PCR products (Section 2.16.2) generated from λMC11 or λMC12 (Section 2.15) and in this way complete sequences of both *pyr*4 genes were obtained (Fig. 12, see also Appendix 3). A six frame translation of these nucleotide sequences was performed using the MAP program of the GCG package. This gave a number of open reading frames (ORFs). Two of these ORFs, one from each λ clone sequenced, showed similarity to amino acid sequences of a number of known *pyr*4 gene products (see Section 3.3.5), confirming that the two sequences that hybridised to *pyr*4 from *C. purpurea* are both *pyr*4. The *pyr*4 from λMC12, λMC14 and λMC20 was designated *pyr*4-1, and that from λMC11 was designated *pyr*4-2.

3.3.3 Identification of Proposed Sites for Translational Start and Transcriptional Initiation of *pyr*4-1 and *pyr*4-2

The inferred translational start site for the two genes was identified by comparisons of OMPD-1 and OMPD-2 to OMPdecarboxylase amino acid sequences from other filamentous fungi (Section 3.3.5). Sequences around the start sites, UCGCAACC**AUG**GCR, are similar to two Kozak sequences which have been proposed for *Neurospora crassa*: CAMM**AUG**GCU (Edelmann and Staben 1994) and CNNNCAM$\cup$**AUG**GC (Bruchez *et al.* 1993), and are also similar to the vertebrate Kozak sequence of GCCRMC**AUG**G (Kozak 1991) (where: N = A, G, C or T; R = A or G; M = C or A; and $\cup$ = a conserved absence of U in this position). Given the context of the proposed translational start site and comparisons of Lp1 *pyr*4 sequences to

Fig. 12. Sequences of two *pyr*4 genes from Lp1.

DNA sequences of the Lp1 *pyr*4-1 and *pyr*4-2 genes and deduced amino acid sequences of OMPD-1 and OMPD-2. For the regions shown nucleotide sequences of both strands were determined. Nucleotides and amino acids are numbered from the putative translational start codon determined by comparisons to known OMPdecarboxylases as outlined in section 3.3. Differences between the two sequences are in bold. Sequences of the primers are underlined; single underlines for primers of identical sequence to that shown, double underlines for primers that are complementary to the sequence shown. Numbers of the primers are shown above their sequences. Where two primers are present the first number corresponds to the primer with the double underlined (complementary) sequence. Nucleotide sequences above are held in the GenBank database under accession numbers U14564 (*pyr*4-1) and U14565 (*pyr*4-2).


mc8
pyr4-1 AGGAACTAAACATGCTTAGAAGTTAGAGGCCTAGCTATATAAGTCTAAGGAAAGGGTAATTATTAC----------ATGTAGGGATTAAGACCCCTTTTCTTACTATCAGAGGTCTTATTAATA----TGGATAACTTACTTAATCTGTC -283
pyr4-2 AGGAACTAAACATGCTTAGAAG-------CCTAGCTATTTAAGTCTAAGGAAAGTGGAATTATTAGGGATTATGTTATCAAGGG-TTAAACCCCCTTTT--TACTATTAGAGGGCTTATTAATACATATGGATAACTTACTCAATCTGTC -286
pyr4-1 ---CAGGGCGATTATCCCGTA---ATTGGTGGACGCGAAGGGGGGAAGCTGTGCCCCACTCCCCTTACGAAAATGTTTCTCCGCAGGAGATAGTTGGGGTTTTTTTTTTTCTTCAT--CCTTATT-----AAGCTAATTGACTTTTGTTC -147
pyr4-2 TATGTAAGGCGACTATCCTGTATGTAGTGGTAAACGCGAAGGGGGGGAGGTGGACCCCACTCCCCTTATGAAAATGTTTCTCCGCAGG----------TTTTTTTTTTTCTTCTCTACCTCATAATAAGAAGCTAATTGACTTTTGTTT -147
pyr4-1 ATGAAAACATCATCTGAGTATTGACCCTACATTGTTTCACCGTCACATTTTTTTTGCCGAGGAACTTGTCTACTTGCGCAAATTCAATATATCTTGACAAAGCCTTTTACGTACCAAACGATCCAAATATTGTGGCGAGATCGCAACCATG 3
pyr4-2 ATGAAACCATCATCTGGGTATTGACCCTGCATTGTTTCATCGTCAAATTTTATTGTCAAGAAACTTATTGTCTTGCGCAAATTCAATATATCTTGACAAAGCCTTTTACGTACCAAACGATCCAAATATTGTGGCGAGATCGCAACCATG 3
M 1
M 1
mc4&5
pyr4-1 GCGCCGCACCAAACCCTCAAGGCGACTTACGCCTCGAGAGCTCAGAGTGCCACACACCCGCTCAACGCCTATCTATTCAAGCTTATGGACTTGAAGGCTTCCAACCTCTGTTTGAGTGCTGATGTCGCGACTGCCCGGGAACTTCTTTAC 153
pyr4-2 GCACCGCACCAAACCCTCAAGGCGACTTACGCCTCGAGAGCTCAGAGTGCCACACACCCGCTCAACGCCTATCTATTCAAGCTTATGGACTTGAAGGCGTCCAACCTCTGTTTGAGTGCTGATGTCGCGACTGCCCGGGAACTTCTTTAC 153
OMPD-1 A P H Q T L K A T Y A S R A Q S A T H P L N A Y L F K L M D L K A S N L C L S A D V A T A R E L L Y 51
OMPD-2 A P H Q T L K A T Y A S R A Q S A T H P L N A Y L F K L M D L K A S N L C L S A D V A T A R E L L Y 51
pyr4-1 TTTGCCGACAAGCTTGGCCCGTCTATTGTTGTTCTCAAGACACATTATGATATGGTCTCCGGCTGGGACTTCCACCCGCAAACTGGCACTGGCGCAAAGTTGGCGTCGTTAGCTCGTCGCCATGGATTCTTGATATTCGAAGATCGCAAG 303
pyr4-2 TTTGCCGACAAGCTTGGCCCGTCTATCGTTGTTCTCAAGACGCACTATGATATGGTCTCCGGCTGGGACTTCCACCCGCAAACTGGCACTGGCGCAAAGTTGGCGTCGTTAGCTCGTCGACATGGATTCTTGATTTTCGAAGATCGCAAG 303
OMPD-1 F A D K L G P S I V V L K T H Y D M V S G W D F H P Q T G T G A K L A S L A R R H G F L I F E D R K 101
OMPD-2 F A D K L G P S I V V L K T H Y D M V S G W D F H P Q T G T G A K L A S L A R R H G F L I F E D R K 101
mc6
mc7
pyr4-1 TTCGCTGACATTGGTAACACGGTGGAGTTGCAGTACATTGGCGGTTCTGCACGCATCATCGAGTGGGCACACATAGTCAATGTTAACATGGTTCCCGGAAAGGCGTCTGTCACTTCCCTGGCAAATGCTGCTACCCGGTGGTTCGAACGG 453
pyr4-2 TTCGCTGACATTGGTAACACGGTGGAGTTGCAGTACATTGGCGGTTCTGCGCGCATCATCGAGTGGGCACACATAGTCAATGTTAACATGGTTCCCGGAAAGGCGTCTGTCACTTCCCTGGCAAATGCTGCTACCCGGTGGTTCGAACGG 453
OMPD-1 F A D I G N T V E L Q Y I G G S A R I I E W A H I V N V N M V P G K A S V T S L A N A A T R W F E R 151
OMPD-2 F A D I G N T V E L Q Y I G G S A R I I E W A H I V N V N M V P G K A S V T S L A N A A T R W F E R 151
pyr4-1 TACCCGTATGAAGTGAAGACGTCAGTTTCCGTGGGCACACCGACTCCGGAAGAATTTGAAGAAAATGACGCGGGGTCCAGCGGCCGGGACGAAGCTAACGCACAAGACATCTCGGCGGCCAGACGATGGTCGTAAGGGGAGCATCGTCTCC 603
pyr4-2 TACCCGTATGAAGTGAAGACGTCAGTTTCCGTGGGCACACCGACTGCGGAAGACTTTGAAGAAAATGACGCGGGGTCCAGCGGCCGGGACGAAGCTAATGGCAATACATCTCGGCGGCCAGACGATGGTCGTAAGGGGAGCATCGTCTCC 603
OMPD-1 Y P Y E V K T S V S V G T P T P E E F E E N D A G S S G R D E A N D K T S R R P D D G R K G S I V S 201
OMPD-2 Y P Y E V K T S V S V G T P T A E D F E E N D A G S S G R D E A N G N T S R R P D D G R K G S I V S 201
mc2&3
pyr4-1 GTCACGACAGTAACACAGCAGCAATACGAACCCGTCAACTCTCCTCGACTGACCAAAAGCATGACTGGCGGGGATGAGGTTCTATTTGCTGGCATCGAGGAGGCACCTATGGCGCGAGGTCTACTCATTCTGGCACAGATGTCCTCGGCG 753
pyr4-2 GTCACGACAGTAACACAG--CAATACGAACCCGTCAACTCTCCCCGACTGACCAAAAGCATGACTGGTGGGGATGAGGTTCTGTTTGCTGGCATCGAGGAGGCACCTATGGCGCGAGGTCTACTCATTCTGGCCAGATGTCCTCGGCG 750
OMPD-1 V T T V T Q Q Q Y E P V N S P R L T K S M T G G D E V L F A G I E E A P M A R G L L I L A Q M S S A 251
OMPD-2 V T T V T Q - Q Y E P V N S P R L T K S M T G G D E V L F A G I E E A P M A R G L L I L A Q M S S A 250
pyr4-1 GGTAATTTTATGAATAAGGAGTATACACAAGCTTGCGTGGAGGCTGCCAGAGAACACAAAGATTTTGTCATGGGATTTGTATCACAAGAGACGCTGAATACGGAACCCGATGACAAATTTATTCACATGACGCCAGGCTGCCAGCTGCCA 903
pyr4-2 GGGAACTTTATGAATAAGGAGTATACACAAGCTTGCGTGGAGGCTGCCAGAGAACACAAAGACTTTGTAATGGGATTTGTATCACAAGAGACGTTGAATACGGAACTCGATGACGAATTTATTCACATGACGCCAGGCTGCCAGCTGCCG 900
OMPD-1 G N F M N K E Y T Q A C V E A A R E H K D F V M G F V S Q E T L N T E P D D K F I H M T P G C Q L P 301
OMPD-2 G N F M N K E Y T Q A C V E A A R E H K D F V M G F V S Q E T L N T E L D D E F I H M T P G C Q L P 300
pyr4-1 CCCGAAGACGAGGATCAGAACGGCACGGTCCAGGGCGACGGAAAAGGCCAGCAGTATAATACACCACAAAAGATTATCGGCGTAGCTGGAGCGGACATCGTCATTGTCGGCCGTGGGATCCTTAAAGCAGGGGACCCCGAGGGCGAAACG 1053
pyr4-2 CCCGAAGACGAGGATCAAAACGGCACAGTCCAGGGCGACGGAAAAGGCCAGCAGTATAATACACCACAAAAGATTATCGGCGTAGCTGGAGCGGACATCGTCATTGTCGGCCGTGGGATCCTCAAAGCAGGGGACCCCGAGGGCGAAACG 1050
OMPD-1 P E D E D Q N G T V Q G D G K G Q Q Y N T P Q K I I G V A G A D I V I V G R G I L K A G D P E G E T 351
OMPD-2 P E D E D Q N G T V Q G D G K G Q Q Y N T P Q K I I G V A G A D I V I V G R G I L K A G D P E G E T 350
pyr4-1 GAGCGATATCGCTCTGCGGCTTGGAAGGGTTACACTGAGAGGGTTCGCTAGCAACTTGAGCTAGGTGGCTGGCCATTGGTTCTGAAAATTTTGCTTGAAACTAACCTGGCATTTTGAAGGAAGAAGCATAAACATGGGGAAAATAGGAGG 1203
pyr4-2 GAGCGATATCGCTCTGCGGCTTGGAAGGGTTACACTGAGAGGGTTCGCTAGCAACTTGAGCCAGGTGGCTGGTCATTGGTTCTGAAAATTTTGCTTGAAACTAACCTGACGTTTTGAAGGAAGAAGCACAAACATGGGAAAAATAGGAGG 1200
OMPD-1 E R Y R S A A W K G Y T E R V R * 367
OMPD-2 E R Y R S A A W K G Y T E R V R * 366
mc1
pyr4-1 TACGCATCATTCTCTGTTTCGGCATCATGCGTTACGCTTAGCTAATTATGGAATACAAGCATCCTTCCCCCCGATCCTTTTTGACAACAGCCAACTTCCTAATCTGCAATGGCATTAGGCTTTGAAGA 1331
pyr4-2 TACGCATCATTCTCCGTTTCGGCATCATGTGTTACGCTTAGCTAATTATGGAATACAAGCATCCTTCCCCCCAATTCTCTTTGACAACAATCAACTTCCTAACCCGTAATGGCATTAGGCTTTGAAGA 1328

other known OMPdecarboxylase sequences (Section 3.3.5), it seems likely that this is the translational start site for *pyr*4-1 and *pyr*4-2.

Inspection of the sequences revealed that within the 5' non-coding regions of both genes there are pyrimidine-rich sequences centred about a poly-T stretch at about -185. Pyrimidine-rich sequences are found in a number of fungal promoters, with the site of transcriptional initiation by RNA polymerase II often found within or immediately downstream of them (Gurr *et al.* 1987). However the significance of the pyrimidine rich sequences identified here awaits experimental elucidation.

3.3.4 Comparisons of *pyr*4-1, *pyr*4-2, OMPD-1 and OMPD-2

3.3.4.1 Sequence Comparisons

A total of 1750 bp of the *pyr*4-1 region and 1754 bp of the *pyr*4-2 region were sequenced (Fig. 12). The two sequences were aligned manually using the LINEUP sequence editor of the GCG package. After the introduction of alignment gaps into the sequences the comparison of the two nucleotide sequences spanned 1778 bp. There were a total of 90 differences, counting insertions or deletions (indels) as one difference, between the two sequences (95.4% identity), the majority of these being in non-coding regions. The 5' non-coding sequences had 88.5% identity (45 differences) and 3' non-coding sequences had 93.0% identity (16 differences). The *pyr*4-1 ORF spans 1104 nucleotides and codes for a 367 amino acid polypeptide with a predicted MW of 40,202 Daltons (OMPD-1). The *pyr*4-2 ORF is 1101 nucleotides long, coding for a 366 amino acid polypeptide with a predicted MW of 39,979 Daltons (OMPD-2). The difference in length between the two sequences is the result of an indel of 3 base pairs (bp) at nucleotide positions 622-624 in the *pyr*4-1 sequence. The ORFs have 97.5% identity at the nucleotide level (29 differences, counting the indel at nucleotide 622 as only one difference). Of these differences 22 were synonymous, the remaining 7 resulting in amino acid changes, giving 98.4% identity (98.9% similarity) between the two polypeptides.

3.3.4.2 Comparisons of GC Content of *pyr*4-1 and *pyr*4-2

GC content of the regions sequenced was determined with the COMPOSITION programme of GCG. GC content of both sequences is almost identical over the entire region sequenced. The *pyr*4-1 region has 49.0% GC over 1750 bp of sequence and the *pyr*4-2 region has 48.6% GC over 1754 bp. The *pyr*4-1 ORF has 53.7% GC, and that of *pyr*4-2 has 54.2% GC. Non-coding sequences showed a preference to AT. The *pyr*4-1 5' non-coding sequence has a GC content of 39.9% and 5'

non-coding sequence of *pyr*4-2 has 37.3% GC. The *pyr*4-1 3' non-coding sequence has 43.2% GC and that of *pyr*4-2 has 42.3% GC.

3.3.5 Comparisons of OMPD-1 and OMPD-2 to Other OMPdecarboxylase Sequences

3.3.5.1 Similarities of OMPD-1 and OMPD-2 to OMPdecarboxylase Sequences from the Pyrenomycetes and their Imperfect Relatives

When compared to translations of *pyr*4 genes from other fungi of the subclass Pyrenomycetes, using the GAP program of the GCG package (Devereux *et al.* 1984), amino acid sequences of OMPD-1 and OMPD-2 both showed 86.6% similarity to the *Cephalosporium acremonium* sequence (Vian and Penalva 1989), 89.3% (OMPD-1) and 87.7% (OMPD-2) similarity to the *Trichoderma reesei* sequence (Berges *et al.* 1990; Smith *et al.* 1991), 86.9% (OMPD-1) and 87.2% (OMPD-2) similarity to the *Trichoderma harzianum* sequence (Heidenreich and Kubicek 1994) and 76.1% (OMPD-1) and 76.4% (OMPD-2) similarity to the *Neurospora crassa* sequence (Newbury *et al.* 1986).

3.3.5.2 Comparisons of OMPD-1 and OMPD-2 to OMPdecarboxylase Sequences from Filamentous Ascomycetes

A PILEUP of the OMPdecarboxylase amino acid sequences from *Aspergillus niger* (Wilson *et al.* 1988), *Penicillium chrysogenum* (Cantoral *et al.* 1988), *Aspergillus nidulans* (Oakley *et al.* 1987), *Trichoderma reesei* (Berges *et al.* 1990; Smith *et al.* 1991), *Trichoderma harzianum* (Heidenreich and Kubicek 1994), *Cephalosporium acremonium* (Vian and Penalva 1989), *Neurospora crassa* (Newbury *et al.* 1986) and Lp1 was performed with a gap weight of 5.0 and a gap length weight of 0.10 (Fig. 13). Sequences (except those of Lp1) were obtained from GenBank sequence database (release 72.0) or by a search of GenBank (release 82.0) at the National Centre for Biotechnology Information with NCSA Mosaic. Translational start sites and intron splice sites of these sequences were determined using information provided with the sequences. Amino acid sequences were determined using the TRANSLATE program of GCG The dendogram from this comparison (Fig. 14) agrees with the similarities of these sequences given above. It also agrees with the phylogeny of the OMPdecarboxylase genes of these fungi published elsewhere (Radford 1993), with the addition of Lp1 sequences closest to those from *Trichoderma*, and within other Pyrenomycetes and related imperfect fungi. In addition these comparisons showed that there were no gaps within amino acid sequences

Fig. 13. Comparisons of OMPD-1 and OMPD-2 to other OMPD amino acid sequences.

Alignment of the OMPdecarboxylase amino acid sequences from *Aspergillus niger, Penicillium chrysogenum, Aspergillus nidulans, Trichoderma reesei, Trichoderma harzianum, Cephalosporium acremonium, Neurospora crassa* and Lp1. Dashes (-) correspond to alignment gaps, asterisks (*) correspond to invariant sites and dots (.) to conservative changes within the sequences.

```
                                 .         .         .         .         .         .
A. niger        --------MSSKSQLTYTARASKHPNALAKRLFEIAEAKKTNVTVSADVTTTKELLDLAD   52
P. chrysogenum  --------MSSKSQLTYTARAQSHPNPLARKLFQVAEEKKSNVTVSADVTTTKELLDLAD   52
A. nidulans     --------MSSKSHLPYAIRATNHPNPLTSKLFSIAEEKKTNVTVSADVTTSAELLDLAD   52
Lp1 OMPD-1      ------MAPHQTLKATYASRAQSATHPLNAYLFKLMDLKASNLCLSADVATARELLYFAD   54
Lp1 OMPD-2      ------MAPHQTLKATYASRAQSATHPLNAYLFKLMDLKASNLCLSADVATARELLYFAD   54
T. harzianum    ------MASHPTLKTTFAARSEATTHPLTSYLLRLMDLKASNLCLSADVPTARELLYLAD   54
T. reesei       ------MAPHPTLKATFAARSETATHPLTAYLFKLMDLKASNLCLSADVPTARELLYLAD   54
C. acremonium   ------MAPHPTLKSTYSTRAETVTHPLSAYLYKLMDLKASNLCLSADVANARELLHVAD   54
N. crassa       MSTSQETQPHWSLKQSFAERVESSTHPLTSYLFRLMEVKQSNLCLSADVEHARDLLALAD   60
                          .  *          *  . . * .*. .****    .**  **

                        .         .         .         .         .         .
A. niger        RLGPYIAVIKTHIDILSDFS----DETIEGLKALAQKHNFLIFEDRKFIDIGNTVQKQYH  108
P. chrysogenum  RLGPYIAVIKTHIDILSDFS----QETIDGLNALAQKHNFLIFEDRKFIDIGNTVQKQYH  108
A. nidulans     RLGPYIAVLKTHIDILTDLT----PSTLSSLQSLATKHNFLIFEDRKFIDIGNTVQKQYH  108
Lp1 OMPD-1      KLGPSIVVLKTHYDMVSGWDFHPQTGTGAKLASLARRHGFLIFEDRKFADIGNTVELQYI  114
Lp1 OMPD-2      KLGPSIVVLKTHYDMVSGWDFHPQTGTGAKLASLARRHGFLIFEDRKFADIGNTVELQYI  114
T. harzianum    KIGPSIVVLKTHYDMVSGWDFHPDTGTGAKLASLARKHGFLIFEDRKFGDIGHTVELQYT  114
T. reesei       KIGPSIVVLKTHYDMVSGWTSHPETGTGAQLASLARKHGFLIFEDRKFGDIGHTVELQYT  114
C. acremonium   KVGPSIVVLKTHYDMVAGWDFTPETGTGARLAKLARKHGFLIFEDRKFGDIGNTVELQYT  114
N. crassa       KVGPSIVVLKTHYDLITGWDYHPHTGTGAKLAALARKHGFLIFEDRKFVDIGSTVQKQYT  120
                ..** * *.*** *..          *   *  ** .* ********* *** **  **

                        .         .         .         .         .         .
A. niger        RGTLRISEWAHIINCSILPGEGIVEALAQTAS----------------------------  140
P. chrysogenum  NGTLRISEWAHIINCSILPGEGIVEALAQTAQ----------------------------  140
A. nidulans     GGALRISEWAHIINCAILPGEGIVEALAQTTK----------------------------  140
Lp1 OMPD-1      GGSARIIEWAHIVNVNMVPGKASVTSLANAATRWFERYPYEVKTSVSVGTPTPEEFEEND  174
Lp1 OMPD-2      GGSARIIEWAHIVNVNMVPGKASVTSLANAATRWFERYPYEVKTSVSVGTPTAEDFEEND  174
T. harzianum    SGSARIIDWAHIVNVNMVPGKASVASLAQGARRWLERYPCEVKTSVTVGTPTMDQFDDAE  174
T. reesei       GGSARIIDWAHIVNVNMVPGKASVASLAQGAKRWLERYPCEVKTSVTVGTPTMDSFDDDA  174
C. acremonium   QGAARIIEWAHIVNVNMVPGKASVTSLANAAAKWLERLPYEVKTSVTVGTPRNTD-EDDD  173
N. crassa       AGTARIVEWAHITNADIHAGEAMVSAMAQAAQKWRERIPYEVKTSVSVGTPVADQFADEE  180
                 *  ** .**** *     * . *  .                          *

                        .         .         .         .         .         .
A. niger        ----------------------------------------------------------  140
P. chrysogenum  ----------------------------------------------------------  140
A. nidulans     ----------------------------------------------------------  140
Lp1 OMPD-1      AGSSGRDEANDKT------------SRRPDDGRKGSIVSVTTVTQQQYEPVNSPRLTKSM  222
Lp1 OMPD-2      AGSSGRDEANGNT------------SRRPDDGRKGSIVSVTTVT-QQYEPVNSPRLTKSM  221
T. harzianum    DAKDDEPATVND--NGSNMMEKPIYAGRNGDGRKGSIVSITTVT-QQYESAASPRLGKTI  231
T. reesei       DSRDAEPAGAVNGMGSIGVLDKPIYSNRSGDGRKGSIVSITTVT-QQYESVSSPRLTKAI  233
C. acremonium   DEDDGNAGEMERSHTFGLNGNNGVPIKESSDGRKGSIVSVTTVT-QQYESAHSPRLTKTI  233
N. crassa       AEDQVEELRKVVTRETST-------TTKDTDGRKSSIVSITTVT-QTYEPADSPRLVKTI  233

                        .         .         .         .         .         .
A. niger        --------APDFSYGPERGLLILAEMTSKGSLATGQYTTSSVDYARKYKNFVMGFVSTRS  192
P. chrysogenum  --------ATDFPYGSERGLLILAEMTSKGSLATGAYTSASVDIARKYPSFVLGFVSTRS  192
A. nidulans     --------SPDFKDANQRGLLILAEMTSKGSLATGESQARSVEYARKYKGFVMGFVSTRA  192
Lp1 OMPD-1      TGGDEVLFAGIEEAPMARGLLILAQMSSAGNFMNKEYTQACVEAAREHKDFVMGFVSQET  282
Lp1 OMPD-2      TGGDEVLFAGIEEAPMARGLLILAQMSSAGNFMNKEYTQACVEAAREHKDFVMGFVSQET  281
T. harzianum    AEGDESLFPGIEEAPLNRGLLILAQMSSEGNFMTGEYTQACVEAAREHKDFVMGFISQEA  291
T. reesei       AEGDESLFPGIEEAPLSRGLLILAQMSSQGNFMNKEYTQASVEAAREHKDFVMGFISQET  293
C. acremonium   AEEGDMLLAGLEEPPLNRGLLILAQMSSAGNFMNAEYTQACVEAAREHKDFVMGFVSQEA  293
N. crassa       SEDDEMVFPGIEEAPLDRGLLILAQMSSKGCLMDGKYTWECVKAARKNKGFVMGYVAQQN  293
                             ******* *.* *          .*  **    **.*..

                        .         .         .         .         .         .
A. niger        LGEV------------QSEVSSPSDEEDFVVFTTGVNISS-----------KGDKLGQQY  229
P. chrysogenum  LGEV------------ESTEAPAS--EDFVVFTTGVNLSS-----------KGDKLGQQY  227
A. nidulans     LSEV------------LPEQKEES--EDFVVFTTGVNLSD-----------KGDKLGQQY  227
Lp1 OMPD-1      LNT--------------------EPDDKFIHMTPGCQLPPEDEDQ--NGTVQGDGKGQQY  320
Lp1 OMPD-2      LNT--------------------ELDDEFIHMTPGCQLPPEDEDQ--NGTVQGDGKGQQY  319
T. harzianum    LNT--------------------QADDDFIHMTPGCQLPPEDEDQQTNGKVGGDGQGQQY  331
T. reesei       LNT--------------------EPDDAFIHMTPGCQLPPEDEDQQTNGSVGGDGQGQQY  333
C. acremonium   LNS--------------------QPDDDFIHMTPGCQLPPEHEE---DAELRGDGKGQQY  330
N. crassa       LNGITKEALAPSYEDGESTTEEEAQADNFIHMTPGCKLPPPGEE-----APQGDGLGQQY  348
                *                          . *.  * *  .                ** ****

                        .         .         .         .         .
A. niger        QTPASAIG-RGADFIIAGRGIYAAPDPVQAAQQYQKEGWEAYLARVGGN*-  277
P. chrysogenum  QTPQSAVG-RGADFIISGRGIYAAADPVEAAKQYQQQGWEAYLARVGAQ*-  275
A. nidulans     QTPGSAVG-RGADFIIAGRGIYKADDPVEAVQRYREEGWKAYEKRVGL*--  274
Lp1 OMPD-1      NTPQKIIGVAGADIVIVGRGILKAGDPEGETERYRSAAWKGYTERVR*---  367
Lp1 OMPD-2      NTPQKIIGVAGADIVIVGRGILKAGDPEGETERYRSAAWKGYTERVR*---  366
T. harzianum    NTAHKIIGIAGSDIAIVGRGILKASDPVEEAERYRSAAWKAYTERLLR*--  378
T. reesei       NTPHKLIGIAGSDIAIVGRGILKASDPVEEAERYRSAAWKAYTERLLR*--  381
C. acremonium   NTPEKLIGVCGADIVIVGRGILKAGDLQHEAERYRSAAWKAYTERVR*---  377
N. crassa       NTPDNLVNIKGTDIAIVGRGIITAADPPAEAERYRRKAWKAYQDRRERLA*  398
                .*    .   * *  * ****  * *       *   .* .*  *
```

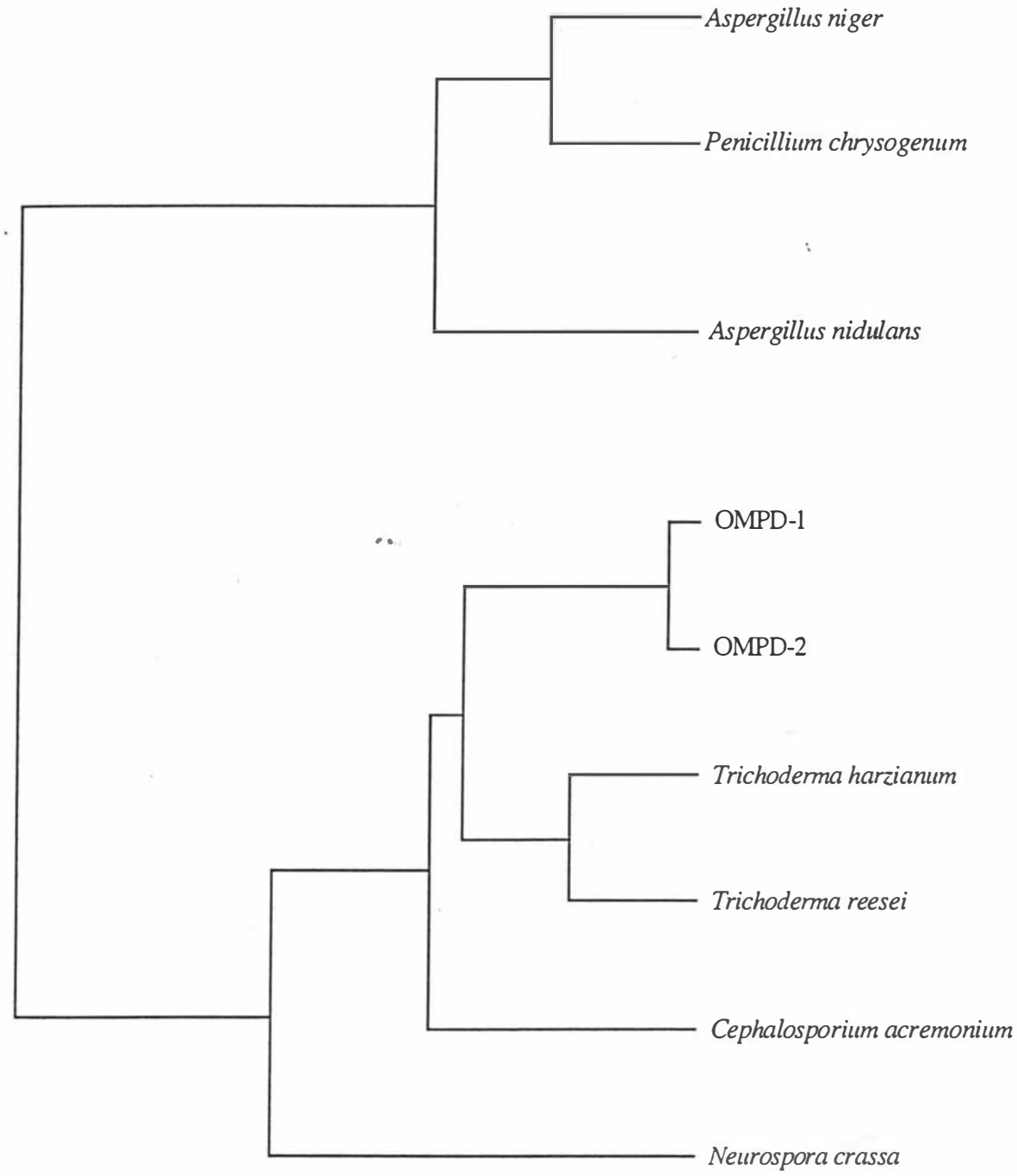


Fig. 14. Dendogram showing relatedness of OMPD-1 and OMPD-2 to OMPdecarboxylase encoding genes from other filamentous Ascomycetes.

Dendogram from PILEUP program of GCG showing clustering relationships used to determine the order of pairwise alignments that together created the final multiple sequence alignment shown in Fig. 13. Distance along the horizontal axis is proportional to the difference between sequences. This dendogram is from a pairwise alignment using the UPGMA clustering strategy hence sequences and clusters of sequences occur in pairs.

of other known OMPdecarboxylase encoding genes along the region of comparison, suggesting that neither *pyr*4-1 or *pyr*4-2 contain introns.

3.3.5.3 Sequence Features Common to OMPD-1, OMPD-2 and Other OMPdecarboxylase Sequences

Both OMPD-1 and OMPD-2 contain a large insert in the middle of the polypeptide. This insert (and a lack of introns) is characteristic of all OMPD sequences available in the GenBank sequence database (release 82.0) from the Pyrenomycetes and related Deuteromycetes, but is not present in OMPdecarboxylase gene sequences from other organisms (Radford 1993). This confirms that imperfect *Acremonium* endophytes have most probably evolved from a sexual species of the Pyrenomycetes as has been previously suggested (Schardl *et al.* 1991). Both OMPD-1 and OMPD-2 contain a highly conserved motif at positions 94-109, which is found in OMPdecarboxylase amino acid sequences of all species characterised to date (Radford 1993). The consensus motif, derived from 17 OMPdecarboxylase sequences in (Radford 1993), plus OMPD-1 and OMPD-2 from Lp1, is F^{19} L^{18}/M^{1} I^{18}/L^{1} F^{19} E^{19} D^{19} R^{19} K^{19} F^{19} $A^{13}/G^{2}/V^{1}/I^{3}$ D^{19} I^{19} G^{19} $N^{17}/H^{1}/S^{1}$ T^{19} V^{19}, where superscript numbers correspond to the number of times individual residues occur within the 19 sequences.

3.3.6 Codon Usage of *pyr*4-1 and *pyr*4-2

Codon usage was measured with the CODONFREQUENCY program of GCG and was clearly biased. There was a preference against A in the third position, but all codons with the exception of stop codons UGA and UAA, were used (Table 4). For *tub*2, the only protein coding gene from the *Epichloë-Acremonium* group of fungi in the GenBank Sequence Database (Release 82.0), codon bias was more extreme (Byrd *et al.* 1990). This is as expected, as highly expressed genes, like *tub*2, show more marked codon bias than genes expressed at lower levels, such as *pyr*4 (Gurr *et al.* 1987). This is thought to be due to the need of highly expressed genes for more common isoaccepting tRNAs.

3.4 EXPRESSION OF *PYR*4-1 AND *PYR*4-2

3.4.1 RT-PCR Analysis of *pyr*4 Transcripts

To determine if the two *pyr*4 genes from Lp1 are expressed, total RNA was isolated (Section 2.18) and analysed by RT-PCR (Section 2.20), using primers mc5 and mc6. These two primers span a *NcoI* site that is unique to *pyr*4-1 and a *SalI* site that is unique to *pyr*4-2 but 4 bp upstream of the position of the *Nco*I site (Fig. 10). These two

Table 4 Combined codon bias table for *pyr*4-1 and *pyr*4-2.

Amino Acid	Codon	Number	Fraction	Amino Acid	Codon	Number	Fraction
Gly	GGG	11.00	0.17	Trp	TGG	8.00	1.00
	GGA	10.00	0.15	STOP	TGA	0.00	0.00
	GGT	12.00	0.18	Cys	TGT	2.00	0.33
	GGC	32.00	0.49		TGC	4.00	0.67
Glu	GAG	24.00	0.46	STOP	TAG	2.00	1.00
	GAA	28.00	0.54		TAA	0.00	0.00
Asp	GAT	15.00	0.36	Tyr	TAT	12.00	0.50
	GAC	27.00	0.64		TAC	12.00	0.50
Val	GTG	8.00	0.16	Leu	TTG	11.00	0.22
	GTA	7.00	0.14		TTA	2.00	0.04
	GTT	14.00	0.28	Phe	TTT	14.00	0.54
	GTC	21.00	0.42		TTC	12.00	0.46
Ala	GCG	23.00	0.32	Ser	TCG	6.00	0.13
	GCA	13.00	0.18		TCA	4.00	0.09
	GCT	25.00	0.34		TCT	12.00	0.26
	GCC	12.00	0.16		TCC	14.00	0.30
Arg	AGG	2.00	0.05	Arg	CGG	12.00	0.30
	AGA	4.00	0.10		CGA	7.00	0.17
Ser	AGT	4.00	0.09		CGT	6.00	0.15
	AGC	6.00	0.13		CGC	9.00	0.22
Lys	AAG	27.00	0.75	Gln	CAG	18.00	0.58
	AAA	9.00	0.25		CAA	13.00	0.42
Asn	AAT	15.00	0.52	His	CAT	3.00	0.19
	AAC	14.00	0.48		CAC	13.00	0.81
Met	ATG	20.00	1.00	Leu	CTG	10.00	0.20
Ile	ATA	3.00	0.09		CTA	5.00	0.10
	ATT	14.00	0.44		CTT	9.00	0.18
	ATC	15.00	0.47		CTC	12.00	0.24
Thr	ACG	16.00	0.30	Pro	CCG	14.00	0.41
	ACA	16.00	0.30		CCA	7.00	0.21
	ACT	16.00	0.30		CCT	3.00	0.09
	ACC	6.00	0.11		CCC	10.00	0.29

unique restriction sites are the result of a point mutational difference between *pyr*4-1 and *pyr*4-2 at nucleotide position 273. Digestion of RT-PCR product from *pyr*4-1 transcripts with *Nco*I will give 271 bp and 53 bp products while the *pyr*4-2 product will remain uncut thereby demonstrating if *pyr*4-1 transcripts are present. Digestion of RT-PCR product from *pyr*4-2 with *Sal*I will give 267 bp and 57 bp products while the *pyr*4-1 RT-PCR product will remain uncut thereby demonstrating if *pyr*4-2 transcripts are present. The PCR on reverse transcribed RNA from Lp1 (Fig. 15) shows a product which runs at 324 bp (lane 3), the expected size of an RT-PCR product from either *pyr*4 (Fig. 10). This product is absent in negative control samples that either lacked RNA (lane 1) or reverse transcriptase (lane 2). Digestion (Section 2.9) of the 324 bp product with either *Nco*I (lanes 5 and 6) or *Sal*I (lanes 7 and 8) gave two fragments of expected sizes showing that both *pyr*4-1 and *pyr*4-2 transcripts are present in Lp1. Digestion of the 324 bp RT-PCR product with both *Nco*I and *Sal*I (lanes 9 and 10) gave the expected two bands but some of the product remains undigested. This is most likely due to heteroduplex formation during PCR; a product that will not be recognised by either restriction enzyme. These results demonstrate that both *pyr*4 genes from Lp1 are transcribed in mycelia grown on 2.4% potato dextrose agar. In addition, as both transcripts were amplified with the same set of primers in the same reaction, and the sequences are nearly identical (6 differences over 324 bp), the amplification kinetics of the two *pyr*4 genes should be identical, as in competitive RT-PCR (Foley *et al.* 1993; Gilliand *et al.* 1990b). Therefore, relative levels of the two RT-PCR products should reflect relative levels of the two *pyr*4 transcripts (Foley *et al.* 1993; Gilliand *et al.* 1990a). The intensity of digested RT-PCR products for *pyr*4-1 (lanes 4 and 5) and *pyr*4-2 (lanes 6 and 7) are similar suggesting that transcripts from both genes are present at similar levels.

3.4.2 Northern Analysis of *pyr*4 Transcripts

It is possible that although transcripts spanning the region amplified by primers mc5 and mc6 are present full length transcripts are not being produced. To test this a northern blot of total RNA used for RT-PCR analysis from Lp1 was hybridised (Section 2.19) to a PCR product from *pyr*4-1 generated with primers mc1 and mc5 (Section 2.15). This PCR product hybridises to both *pyr*4-1 and *pyr*4-2 (data not shown). Fig. 16 shows that only one transcript (of approximately 1.4 kb) is detected from the *pyr*4 genes in Lp1. Given that the two transcripts are present at similar levels (Section 3.4.1) full length messages must be produced from both genes. The highly conserved sequences of the two *pyr*4 ORFs from Lp1 (Fig. 12) made it impossible to distinguish the ORFs on Southern blots (data not shown) and by inference the two transcripts on northern blots.

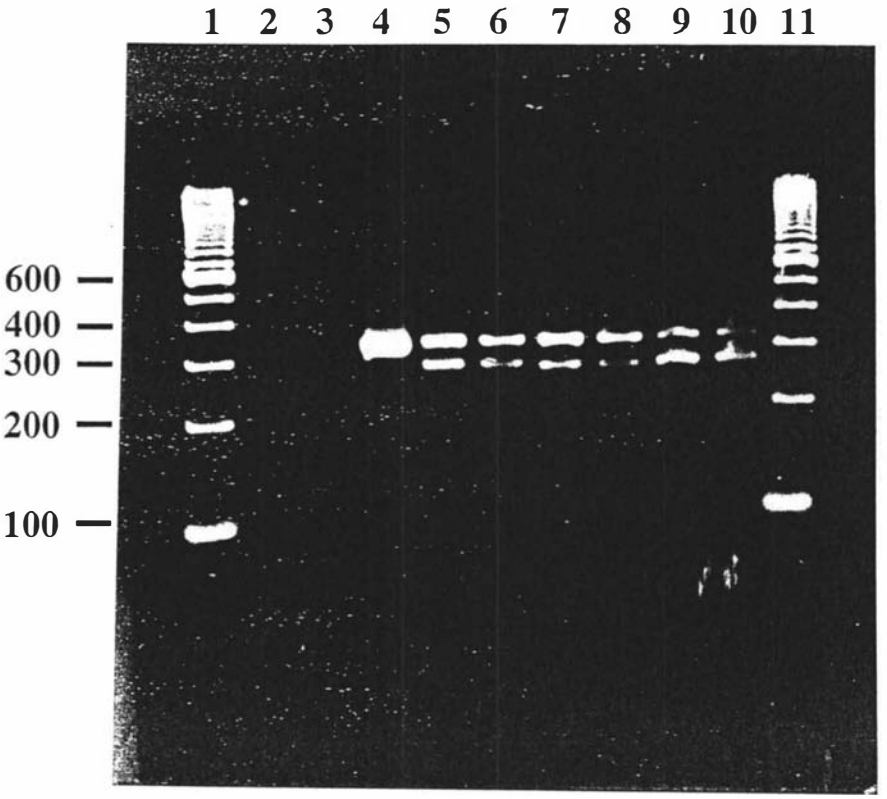


Fig. 15. Identification of transcripts from *pyr*4-1 and *pyr*4-2 in total RNA.

RT-PCR analysis of total RNA from Lp1. All reactions were amplified by PCR as outlined below, with the following templates. Lane 2: No RNA. Lane 3: No reverse transcriptase. Lane 4: Reverse transcribed total RNA from Lp1. Lanes 5-10 contain RT-PCR product shown in lane 4 but digested and loaded as follows. Lane 5: *Nco*I digested RT-PCR product. Lane 6: as for lane 5 but half the quantity. Lane 7: *Sal*I digested RT-PCR product. Lane 8: as for lane 7 but half the quantity. Lane 9: *Nco*I and *Sal*I double digested RT-PCR product. Lane 10: as for lane 9 but half the quantity. Lanes 1 and 11 contain the 100 bp ladder from BRL, the sizes of the bands indicated are in bp.

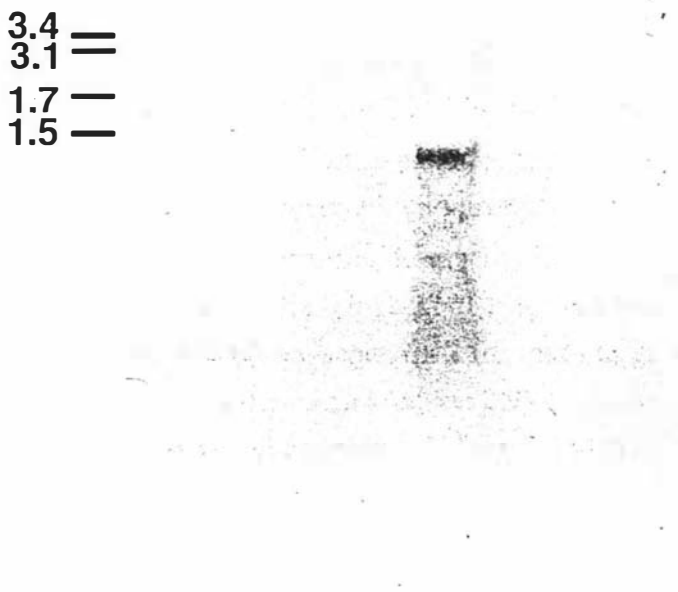


Fig. 16. Identification of transcripts from *pyr*4-1 and *pyr*4-2.

Hybridisation of *pyr*4-1 to northern blot of total RNA from Lp1. Autoradiograph of a northern blot of total RNA from Lp1 hybridised with a PCR product from the *pyr*4-1 gene of Lp1 generated with primers mc1 and mc5 and labelled with [α-^{32}P]dCTP. Positions of the nuclear and mitochondrial small and large rRNA subunits are shown by bars. Sizes indicated are those determined for yeast (Sriprakash and Clark-Walker, 1980).

3.5 COMPLEMENTATION OF AN *ASPERGILLUS NIDULANS* URACIL AUXOTROPH BY *PYR*4-1 AND *PYR*4-2

3.5.1 Subcloning Suitable Genomic Fragments for Complementation

To test if both *pyr*4 genes produce functional polypeptides it was first necessary to subclone suitable genomic fragments containing *pyr*4 which could then be used for complementation analysis. For *pyr*4-1 the 9.7 kb *Sal*I fragment from λMC14 was subcloned (Section 2.11) into pUC118 to give construct pMC7 (Table 1). Digestion of pMC7 with *Bam*HI and *Sal*I (Section 2.9) gave fragments (Table 5) consistent with the map shown (Fig. 17). For *pyr*4-2 no suitable *Bam*HI, *Eco*RI or *Sal*I sites were available for subcloning the complete gene (Fig. 10). Analysis of *pyr*4-2 sequence with the MAP program of the GCG package (Devereux *et al.* 1984) revealed a number of restriction enzymes that lacked sites within this region, these included *Bgl*II, *Cla*I, *Pst*I, *Stu*I and *Xba*I. λMC11 was digested (Section 2.9) with each of these enzymes, analysed by gel electrophoresis (Fig. 18A) and a Southern blot of this gel was hybridised (Section 2.12) to a PCR product from *pyr*4-2 generated with primers mc1 and mc8 from λMC11 DNA (Section 2.15, Fig. 18B). This identified fragments for each of these five enzymes that contained the entire *pyr*4 gene. The 8.9 kb *Xba*I fragment was subcloned (Section 2.11) into the *Xba*I site of pAN8-1 (see Appendix 2 for map) to give construct pMC11 (Table 1). pMC11 was mapped by digestion (Section 2.9) of this clone with *Bam*HI and *Sal*I. These digests gave the expected fragments (Table 5) and are consistent with maps shown in Figs. 10 and 17.

3.5.2 Transformation of *A. nidulans* with *pyr*4-1 and *pyr*4-2

Plasmids pMC7 and pMC11, along with pGM32, containing *Neurospora crassa pyr*4, were transformed into a uracil requiring (*pyr*G$^-$) mutant of *Aspergillus nidulans* (strain 1-85, Table 1, Section 2.22). A negative control with no DNA (water only) was also performed. The results of this transformation are shown in Table 6 and demonstrated that both *pyr*4-1 and *pyr*4-2 encode functional OMPdecarboxylases. pMC11 had a lower transformation frequency than either pMC7 or pGM32, this is probably due to difference in quality of DNA used for different transformations.

3.5.3 Molecular Analysis of URA$^+$ Transformants of *A. nidulans*

To ensure that putative pMC7 and pMC11 transformants contain plasmid DNA, genomic DNA of five transformants was digested (Section 2.9) with *Bam*HI and *Sal*I, enzymes which give characteristic restriction fragments for pMC7 and pMC11 (see Fig. 17 for map). Southern blots of these digests were probed with [α-^{32}P]dCTP-

Table 5 Sizes of pMC7 and pMC11 restriction fragments.

Plasmid	Restriction Digest	Fragment Size (kb)
pMC7	*Bam*HI	5.8, 4.5, 1.6, 1.0
	*Sal*I	9.7, 3.2
pMC11	*Bam*HI	7.4, 3.4, 2.4, 1.4, 0.3
	*Sal*I	8.4, 4.4, 2.1

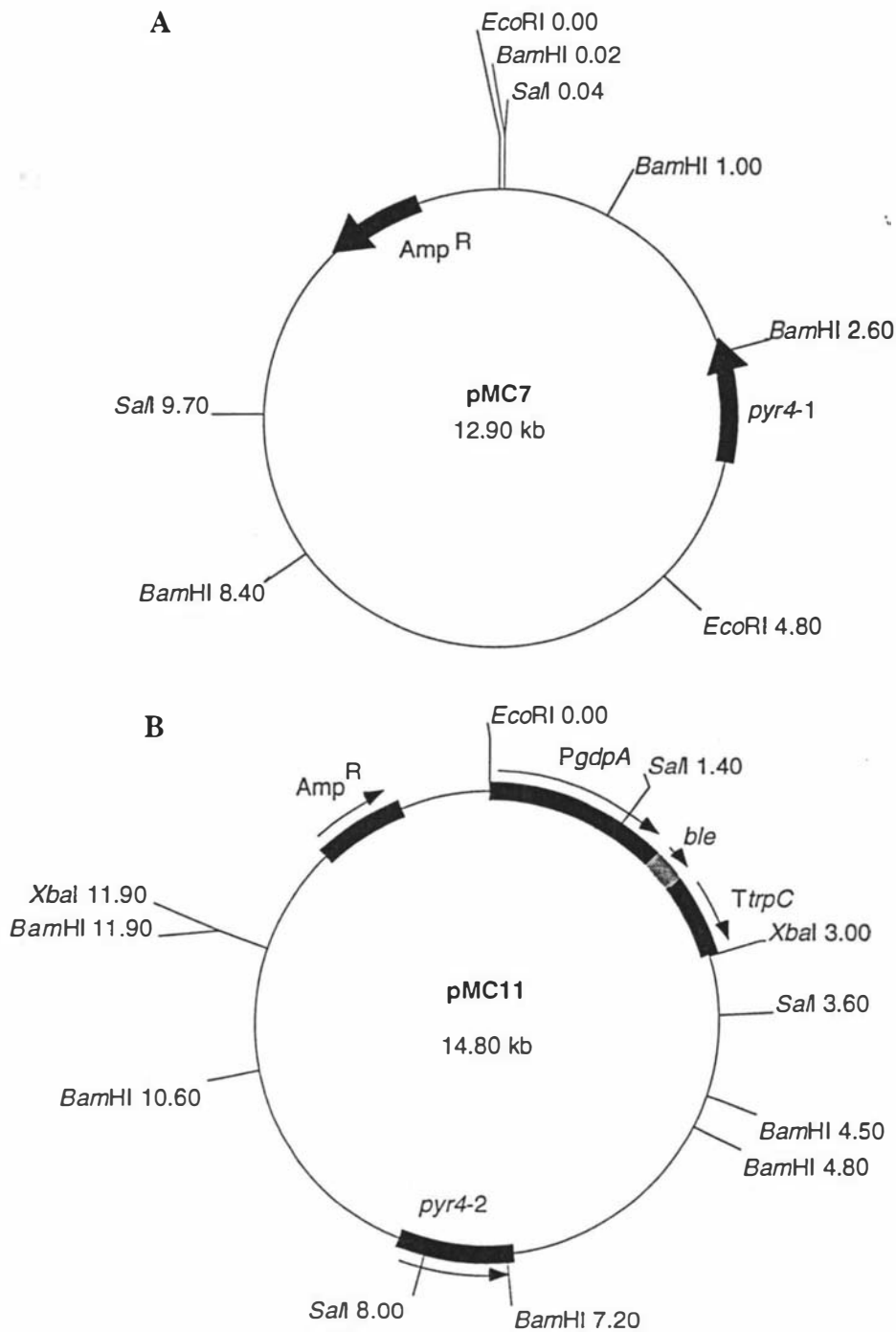


Fig. 17A-B. Restriction maps of pMC7 and pMC11.

A Map of plasmid pMC7 showing sites for *Bam*HI, *Eco*RI and *Sal*I and positions of *pyr*4-1 and Amp^R genes **B** pMC11 showing sites for *Bam*HI, *Eco*RI, *Sal*I and *Xba*I and positions of *pyr*4-2, Amp^R and *ble* genes.

Fig. 18A-B. Identification of restriction fragments from λMC11 suitable for subcloning the complete *pyr*4 gene.

A λMC11 DNA digested with *Bgl*II (lane 3), *Cla*I (lane 4), *Pst*I (lane 5), *Stu*I (lane 6) and *Xba*I (lane 7) and flanked by *Hind*III digested λ DNA (lane 1) and the 1 kb BRL ladder (lane 9). **B** Autoradiograph of a Southern blot of digests shown in A hybridised to an [α-^{32}P]dCTP labelled PCR product from λMC11 generated with primers mc1 and mc8. Numbers on the left of the figure indicate the position and size (kb) of fragments generated by a *Hin*dIII digest of λ DNA.

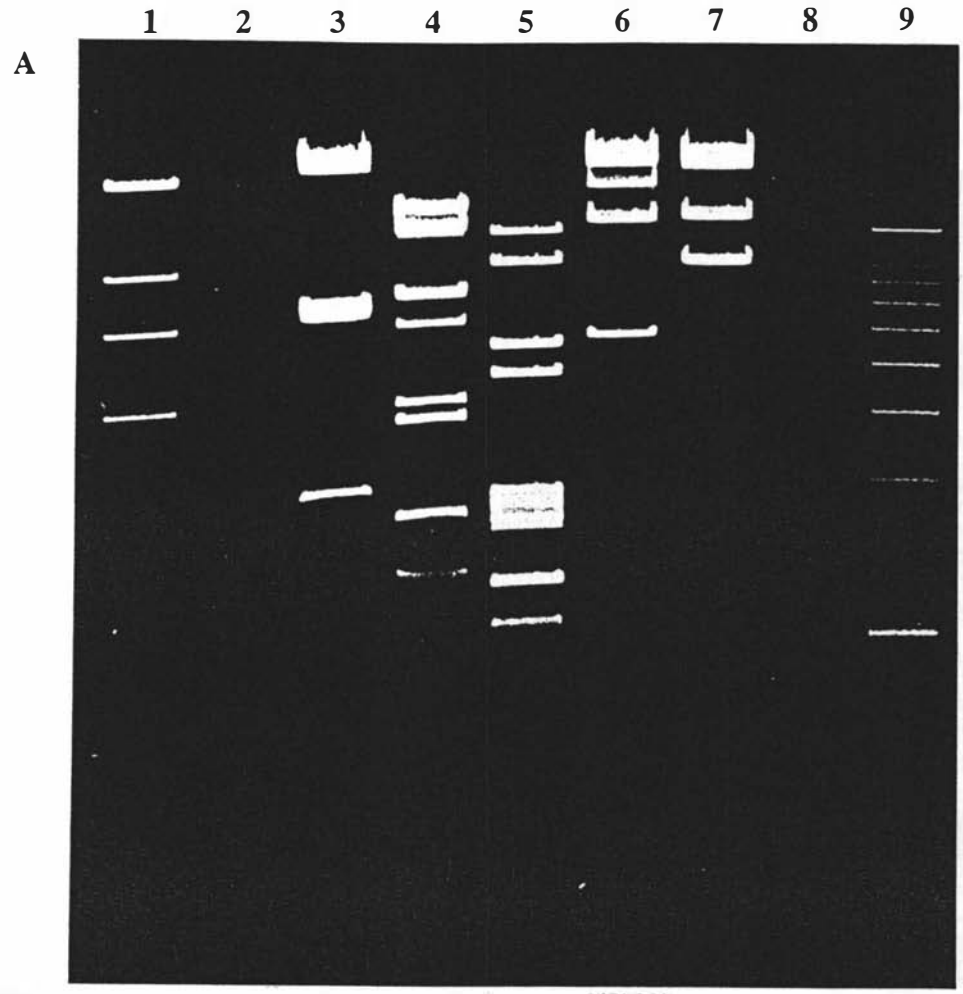

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Table 6 Transformation frequencies of *Aspergillus nidulans* strain 1-85 protoplasts to uracil prototrophy with plasmid constructs containing three different *pyr*4 genes.

Plasmid Construct[a]	Frequency of URA+ Transformants[b]
pMC7	30
pMC11	16[c]
pGM32	35
No DNA	0

[a] All plasmid DNA was purified by a large scale alkali lysis prep (Section 2.5.5) followed by a CsCl/EtBr gradient (Section 2.5.6), except pMC11 which was purified by a small scale alkali lysis prep (Section 2.5.4) followed by a phenol/chloroform extraction (Section 2.6).

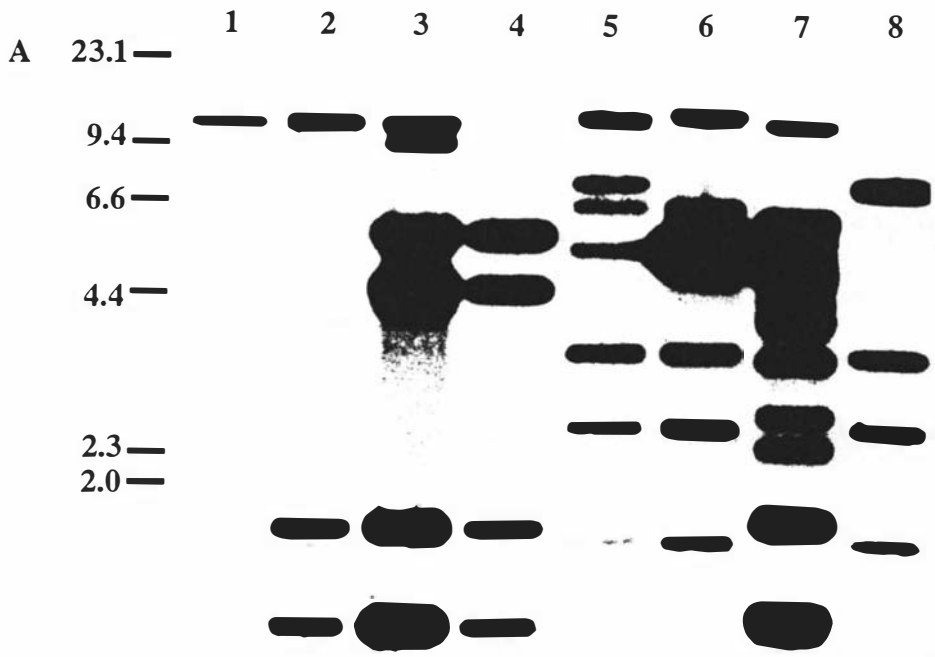
[b] Transformation frequencies are indicated as number of transformants per 10^6 protoplasts per μg DNA. Transformations were performed as in Section 2.22.

[c] pMC11 transformed *A. nidulans* strain 1-85 protoplasts to uracil prototrophy and phleomycin resistant at a frequency of 7 transformants per 10^6 protoplasts per μg DNA.

labelled pMC11 (Section 2.12). Results for the *Bam*HI and *Sal*I digests are shown in Figs. 19A and 19B, and are summarised in Table 7. All transformants were generated with uncut plasmid DNA. Some hybridising fragments were observed in untransformed *A. nidulans* (Fig. 19A and B, lane 1) and fragments of the same size were also present in the transformants, with the exception of the 12 kb *Bam*HI fragment (Fig. 19A) which (presumably) ran as a 13 kb and 13.5 kb *Bam*HI fragment in TrMC11-6 (lane 5) and TrMC11-7 (lane 6) respectively. This may be due to pMC11 integrating into this fragment in these transformants, or other reasons. Whatever the case, these fragments presumably correspond to regions of the *A. nidulans* genome with similarity to pMC11 sequences. TrMC7-2 (lane 2) contained 1.6 and 1.0 kb *Bam*HI fragments characteristic of pMC7. The 5.8 and 4.5 kb *Bam*HI fragments were not detected, these fragments had apparently undergone partial deletion during events associated with transformation or integration and must correspond to faint 3.8 and 1.0 kb *Bam*HI fragments observed (Fig. 19A). The *Sal*I digest was consistent with this (Fig. 19B). TrMC7-7 (lane 3) possessed 5.8, 4.5, 4.0 and 1.6 kb *Bam*HI fragments (Fig. 19A) and 9.7 and 3.2 kb *Sal*I fragments (Fig. 19B) characteristic of head-to-tail tandem repeats of pMC7, as well as 10 and 4.0 kb *Bam*HI (Fig. 19A) and 7.1 and 1.8 kb *Sal*I (Fig. 19B) fragments which presumably correspond to portions of pMC7 plus *A. nidulans* genomic sequences flanking the site of multiple insertions. TrMC11-6 (lane 5) possessed 7.4, 3.4, 2.4 and 1.4 kb *Bam*HI (Fig. 19A) fragments as well as 8.4, 4.4 and 2.1 kb *Sal*I (Fig. 19B) fragments, which are expected for head-to-tail tandem repeats of pMC11 as well as 6.4 and 5.3 kb *Bam*HI (Fig. 19B) fragments which presumably correspond to portions of pMC11 plus *A. nidulans* flanking genomic sequences. However there are no *Sal*I bands present which obviously correspond to portions of pMC11 plus *A. nidulans* DNA (Fig. 19B), this may be explained by these bands being obscured by other bands present in this lane, or other reasons. TrMC11-7 (lane 6) possessed 8.4, 4.4 and 2.1 kb *Sal*I fragments (Fig. 19B) which are expected for head-to-tail tandem repeats of pMC11, however as in TrMC11-6 there are no obvious flanking fragments visible, in addition the 6.0 kb *Sal*I fragment which is observed in all lanes containing *A. nidulans* DNA, is very intense in this transformant. The *Bam*HI digest (Fig. 19A) revealed 3.4, 2.4 and 1.4 kb fragments characteristic of pMC11, however the 7.4 kb *Bam*HI fragment was presumably replaced by a 6.4 kb *Bam*HI fragment, thus this was not entirely consistent with multiple head-to-tail tandem integrants of pMC11, and was inconsistent with data from the *Sal*I digest. In addition, a very intense 5.3 kb *Bam*HI fragment is present, and this probably corresponds to the intense 6.0 kb *Sal*I fragment noted above. TrMC11-8 is more complex than the other transformants, and no *Bam*HI or *Sal*I fragments corresponding in size to fragments observed in pMC11 are present. This is possibly due to major rearrangements within pMC11 during the transformation process. What is obvious from

Fig. 19A-B. Analysis of pMC7 and pMC11 transformants complementing an *Aspergillus nidulans pyrG*$^{-}$ mutation.

Hybridisation of [α-^{32}P]dCTP-labelled pMC11 to 2 μg of total DNA from *A. nidulans* 1-85 and URA^{+} transformants and approximate genomic equivalents of pMC7 and pMC11. **A** Autoradiograph of a Southern blot of *Bam*HI digested total DNA from untransformed *A. nidulans* 1- 85 (lane 1), TrMC7-2 (lane 2), TrMC7-7 (lane 3), pMC7 (lane 4), TrMC11-6 (lane 5), TrMC11-7 (lane 6), TrMC11-8 (lane 7) and pMC11 (lane 8). **B** Autoradiograph of a Southern blot of *Sal*I digests as in **A**. Numbers on the left of the figure indicate the position and size (kb) of fragments generated by a *Hin*dIII digest of λ DNA.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2.3
2.0

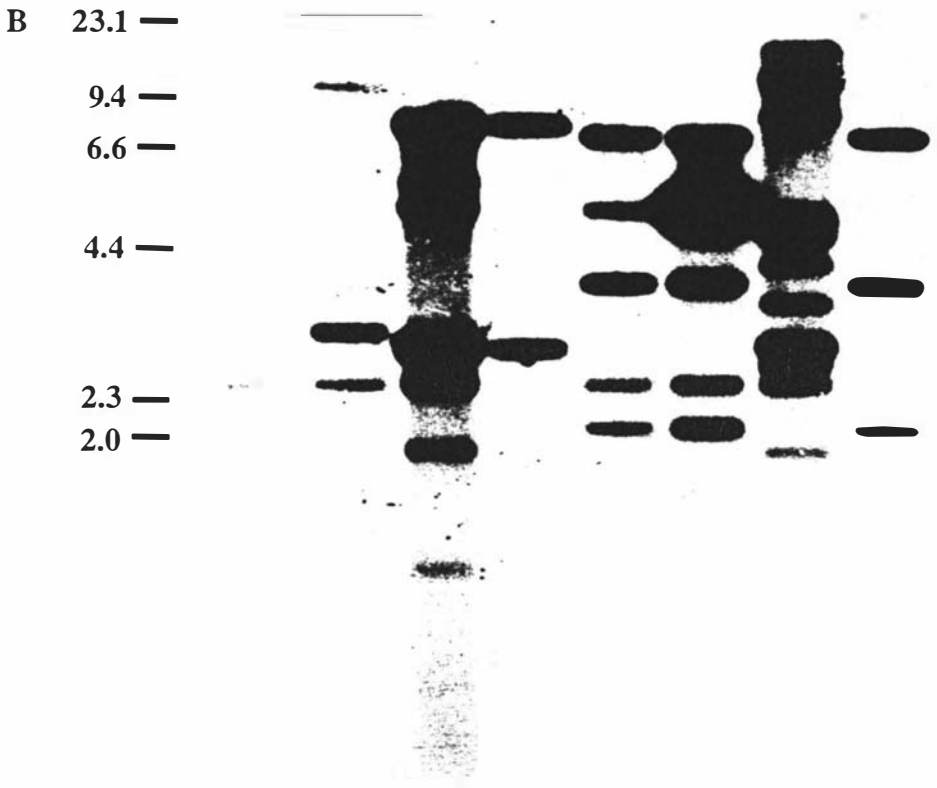

B
23.1
9.4
6.6
4.4
2.3
2.0

Table 7 Sizes of fragments hybridising to pMC11 in untransformed and transformed cultures of *Aspergillus nidulans* strain 1-85.

DNA	Digest	Fragment Sizes (kb)
Aspergillus nidulans 1-85	*Bam*HI	12[a]
(untransformed)	*Sal*I	6.0[a], 2.6[a], 1.0[a]
pMC7	*Bam*HI	5.8, 4.5, 1.6, 1.0
	*Sal*I	9.7, 3.2
TrMC7-2	*Bam*HI	12[a], 3.8, 1.6, 1.0, 0.8
	*Sal*I	14, 6.0[a], 4.2, 3.4, 2.6[a], 1.0[a]
TrMC7-7	*Bam*HI	12[a], 10, 5.8, 4.5, 4.0, 1.6, 1.0
	*Sal*I	9.7, 7.1, 6.0[a], 3.2, 2.6[a], 1.8, 1.0[a]
pMC11	*Bam*HI	7.4, 3.4, 2.4, 1.4
	*Sal*I	8.4, 4.4, 2.1
TrMC11-6	*Bam*HI	13, 7.4, 6.4, 5.3, 3.4, 2.4, 1.4
	*Sal*I	8.4, 6.0[a], 4.4, 2.6[a], 2.1, 1.0[a]
TrMC11-7	*Bam*HI	13.5, 6.4, 5.3[b] 3.4, 2.4, 1.4
	*Sal*I	8.4, 6.0[ab], 4.4, 2.6[a], 2.1, 1.0[a]
TrMC11-8	*Bam*HI	12[a], 6.2, 5.8, 4.8, 3.8, 3.2, 2.6, 2.2, 1.6, 1.0
	*Sal*I	21, 16, 11, 6.0[a], 5.6, 4.7, 4.0, 3.3, 3.0, 2.6[a], 1.8, 1.0[a]

[a] Indicates common bands found in untransformed and transformed *A. nidulans*.

[b] Indicates strong hybridisation signal.

these results (Fig. 19 and Tables 6 and 7) is that plasmid constructs containing both *pyr*4-1 and *pyr*4-2 are able to complement a *pyr*G$^-$ mutation in *Aspergillus nidulans*, and hence both *pyr*4-1 and *pyr*4-2 encode functional OMPdecarboxylases.

3.6 EVOLUTIONARY ORIGINS OF *PYR*4-1 AND *PYR*4-2

3.6.1 Polymorphisms of *pyr*4 in *Acremonium* and *Epichloë* Species

The presence of two *pyr*4 genes was surprising. However, a hypothesis had been put forward that a number of *Acremonium* endophytes were interspecific hybrids, arising by hybridisation of *Epichloë* sp. with either other *Epichloë* spp. or other endophytes (Schardl *et al.* 1994; Tsai *et al.* 1994). Sequences of *rrn* gene segments, including ITS1 and ITS2, from Lp1 and perennial ryegrass choke pathogen, *E. typhina*, had identified *E. typhina* as a possible ancestor of Lp1. Subsequently sequences of *tub*2 5' regions as well as *tub*2 DNA polymorphisms (R. D. Johnson, unpublished data), had identified the *A. lolii* grouping as another possible ancestor, this was supported by examination of isozyme polymorphisms (Schardl *et al.* 1994) and RAPD analysis (D. M. Watt and D. B. Scott, unpublished data). This data strongly suggested that duplicate gene copies which had been identified in Lp1 were a result of interspecific hybridisation, most likely between ryegrass choke pathogen, *E. typhina* (or close relative), and an *A. lolii* (or close relative). To test this a Southern blot of *Bam*HI digested (Section 2.9) total DNA from Lp1, two λ clones (λMC11 and λMC12), *E. typhina* isolate E8, and *A. lolii* isolates Lp5 and Lp9 was hybridised to the 0.65 kb pRS4 *Stu*I fragment (Section 2.12). This revealed that *pyr*4-1 possessed a restriction fragment length polymorphism (RFLP) identical to that of *E. typhina* isolate E8, while *pyr*4-2 possessed an RFLP identical to those of *A. lolii* isolates Lp5 and Lp9 (Fig. 20A). This blot was stripped (Section 2.12.6) and hybridised to λMC11 (Fig. 20B, Section 2.12). This result agreed with those obtained with *C. purpurea pyr*4 . Again *A. lolii* isolates Lp5 and Lp9 possessed RFLPs identical to corresponding portions of λMC11 and Lp1, while the E8 pattern was also found in corresponding regions of λMC12 and Lp1. When this blot was stripped and hybridised to λMC12 (Fig. 20C, Section 2.12) similar patterns were obtained as for λMC11 hybridisation. However, *A. lolii* isolates Lp5 and Lp9 had a band at the same position as *pyr*4-1 from E8, suggesting that *A. lolii* isolates possibly possessed *pyr*4-1, in addition to *pyr*4-2 which had already been identified in these isolates. However, three hybridisation experiments discounted the possibility that this was a *pyr*4 gene, as this 5.8 kb band was not observed when Lp5 and Lp9 were hybridised to: (1) the 0.65 kb pRS4 *Stu*I fragment; (2) λMC11; or (3) the 2.2 kb *Bam*HI-*Eco*RI subclone containing *pyr*4-1 (pMC2, Table 1) from the 5.8 kb *Bam*HI fragment (Fig. 22, see later). Hence this fragment is clearly not *pyr*4-1, but some portion of the Lp5 or Lp9 genome hybridising to

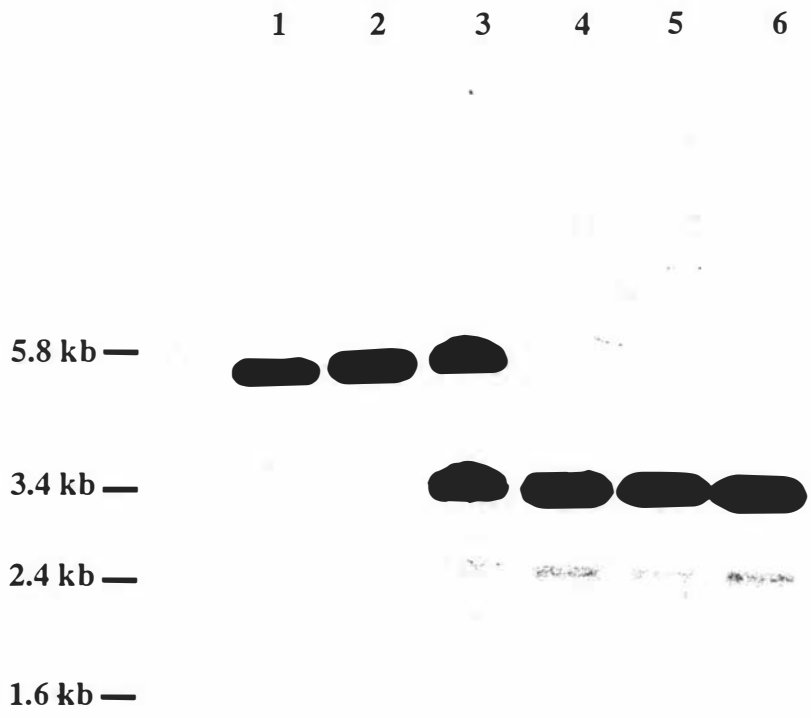
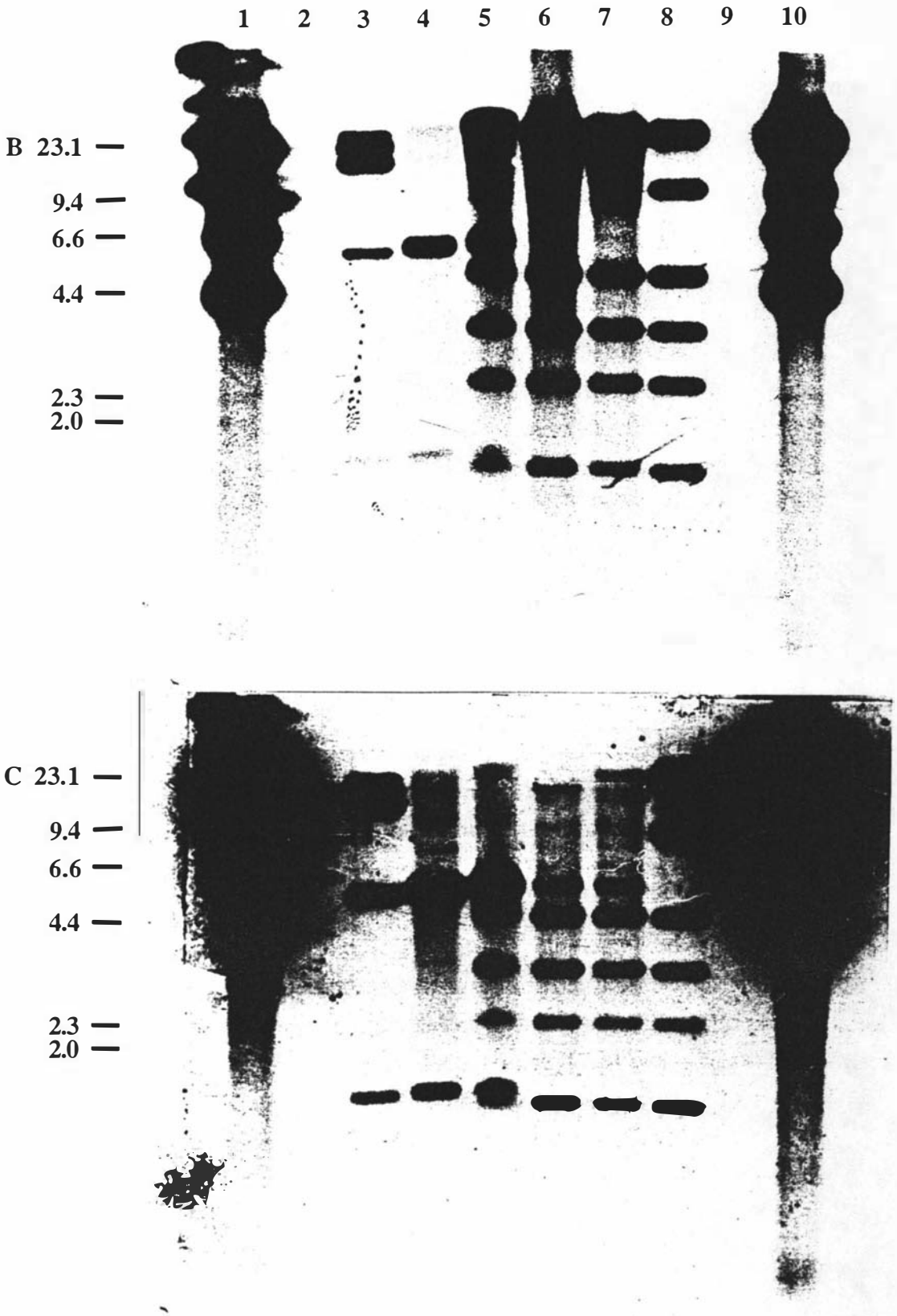


Fig. 20A. Demonstration that *pyr*4-1 and *pyr*4-2 from Lp1 have different evolutionary origins.

Autoradiograph of a Southern blot of *Bam*HI digested total DNA from λ clone MC12 containing *pyr*4-1 from Lp1 (lane 1), *Epichloë typhina* isolate E8 (lane 2), Lp1 (lane 3), *Acremonium lolii* isolates Lp5 (lane 4) and Lp9 (lane 5) and λ clone MC11 containing *pyr*4-2 from Lp1 (lane 6) hybridised to [α-^{32}P]dCTP labelled 0.65 kb pRS4 *Stu*I fragment.

Fig. 20B-C. Demonstration that *pyr*4-1 and *pyr*4-2 from Lp1 have different evolutionary origins.

Autoradiograph of a Southern blot of *Bam*HI digested total DNA from λ clone MC12 containing *pyr*4-1 from Lp1 (lane 3), *Epichloë typhina* isolate E8 (lane 4), Lp1 (lane 5), *Acremonium lolii* isolates Lp5 (lane 6) and Lp9 (lane 7); λ clone MC11 containing *pyr*4-2 from Lp1 (lane 8) hybridised to [α-^{32}P]dCTP-labelled: **B** λMC11; and **C** λMC12. Lanes 1 and 10 contain λ DNA digested with *Hin*dIII. Numbers on the left of the figure indicate the position and size (kb) of fragments generated by a *Hin*dIII digest of λ DNA.


1 2 3 4 5 6 7 8 9 10
B 23.1
9.4
6.6
4.4
2.3
2.0
C 23.1
9.4
6.6
4.4
2.3
2.0

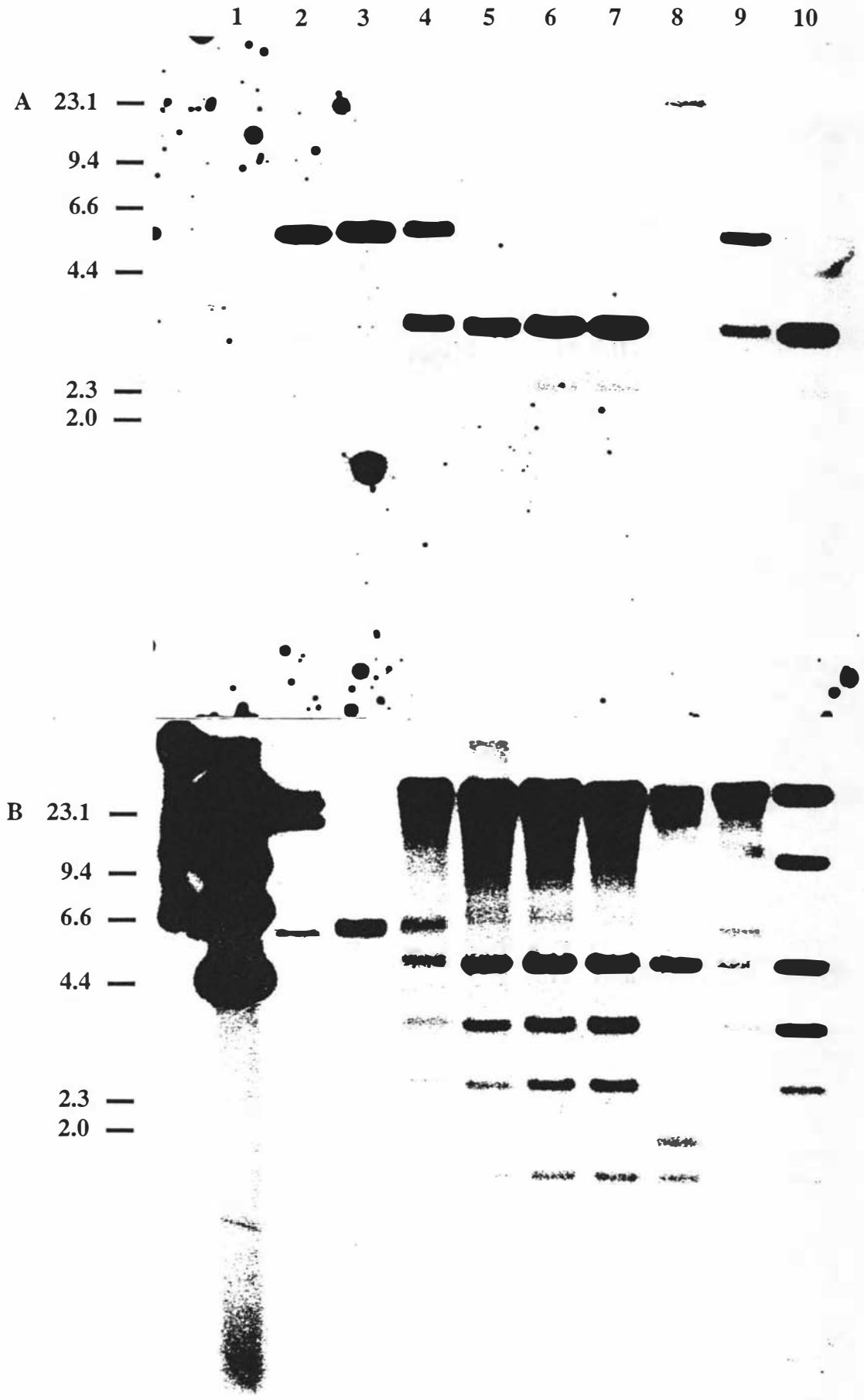
λMC12. A Southern blot containing the same isolates as those in Fig. 20, as well as *A. lolii* isolate Lp6, *E. festucae* E32 and Lp2, a close relative of Lp1 (Christensen *et al.* 1993, and based on isozyme polymorphisms another probable interspecific hybrid) was hybridised to the 0.65 kb pRS4 *Stu*I fragment (Section 2.12). This result suggested that the *A. lolii* isolates were more likely to be ancestors of Lp1 and Lp2 than closely related *E. festucae* (Fig. 21A). RFLPs resulting from hybridisation of λMC11 to this blot supported this assumption (Fig. 21B). An examination of *pyr*4 RFLPs in other *Acremonium* and *Epichloë* spp. was also performed (Fig. 22). Isolates from *E. festucae* isolate E32 (lane 4), *A. lolii* or LpTG-1 isolate Lp5 (lane 5), *E. typhina* isolate E8 (lane 6), LpTG-2 isolate Lp1 (lane 7), *A. uncinatum* or FpTG-1 isolate Tf4 (lane 8), FaTG-2 isolate Tf15 (lane 9), FaTG-3 isolate Tf18 (lane 10) and *A. coenophialum* or FaTG-1 isolate Tf28 (lane 11) were analysed. Based on these RFLPs, isolates from *E. typhina* and *A. lolii* groupings were the most likely ancestors of the Lp1 interspecific hybrid. Other taxa sampled had RFLPs inconsistent with them being likely ancestors of Lp1 (Fig. 22). Interestingly Tf15, Tf18 and Tf28 all had RFLPs similar to Lp5, suggesting they possessed *pyr*4-2, however each of these isolates possessed additional bands which were absent in Lp5 and Lp1.

3.6.2 Demonstrating that Lp1 is Homokaryotic for *pyr*4-1 and *pyr*4-2

As Lp1 had never been single-spore purified (M. J. Christensen, pers. comm.) the Lp1 interspecific hybrid could either be heterokaryotic for two *pyr*4 genes (i.e. contain *pyr*4-1 and *pyr*4-2 in separate nuclei) or homokaryotic for two *pyr*4 genes (i.e. contain *pyr*4-1 and *pyr*4-2 in the same nuclei). If Lp1 was heterokaryotic for *pyr*4 then it is possible that the nuclei would correspond to nuclei of the ancestral species which hybridised to produce Lp1. To test this, single-spored isolates of Lp1 were obtained from M. J. Christensen and analysed for the presence of either one or two copies of *pyr*4. Asexual spores (conidia) of Lp1 had been shown to possess a single nuclei by DAPI staining (Schardl *et al.* 1994), hence if Lp1 was a heterokaryon for two *pyr*4 genes then single-spored isolates would only possess one copy of *pyr*4. However, if Lp1 was a homokaryon for two *pyr*4 genes then single-spored isolates would possess *pyr*4-1 and *pyr*4-2. A Southern blot of *Bam*HI digested (Section 2.9) DNA from a non-single spored isolate of Lp1, and six single spored isolates of Lp1 was hybridised (Section 2.12) to a PCR product from *pyr*4-2 generated with primers mc1 and mc8 (Section 2.15). This demonstrated (Fig. 23) that single-spored isolates and non-single spored isolate possessed identical *pyr*4 RFLPs. Thus, Lp1 is a homokaryon for *pyr*4, containing *pyr*4-1 and *pyr*4-2 within the same nucleus.

Fig. 21A-B. Further evidence for the origins of *pyr*4-1 and *pyr*4-2 in Lp1.

Autoradiograph of a Southern blot of *Bam*HI digested total DNA from λMC12 containing *pyr*4-1 from Lp1 (lane 2), *Epichloë typhina* isolate E8 (lane 3), LpTG-2 isolate Lp1 (lane 4), *Acremonium lolii* (LpTG-1) isolates Lp5 (lane 5), Lp6 (lane 6) and Lp9 (lane 7), *Epichloë festucae* isolate E32 (lane 8), LpTG-2 isolate Lp2 (lane 9) and λMC11 containing *pyr*4-2 from Lp1 (lane 10) hybridised to [α-^{32}P]-labelled: **A** 0.65 kb pRS4 *Stu*I fragment; **B** λMC11. Lane 1 contains λ DNA digested with *Hin*dIII. Numbers on the left of the figure indicate the position and size (kb) of fragments generated by a *Hin*dIII digest of λ DNA.


1 2 3 4 5 6 7 8 9 10
A 23.1
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B 23.1
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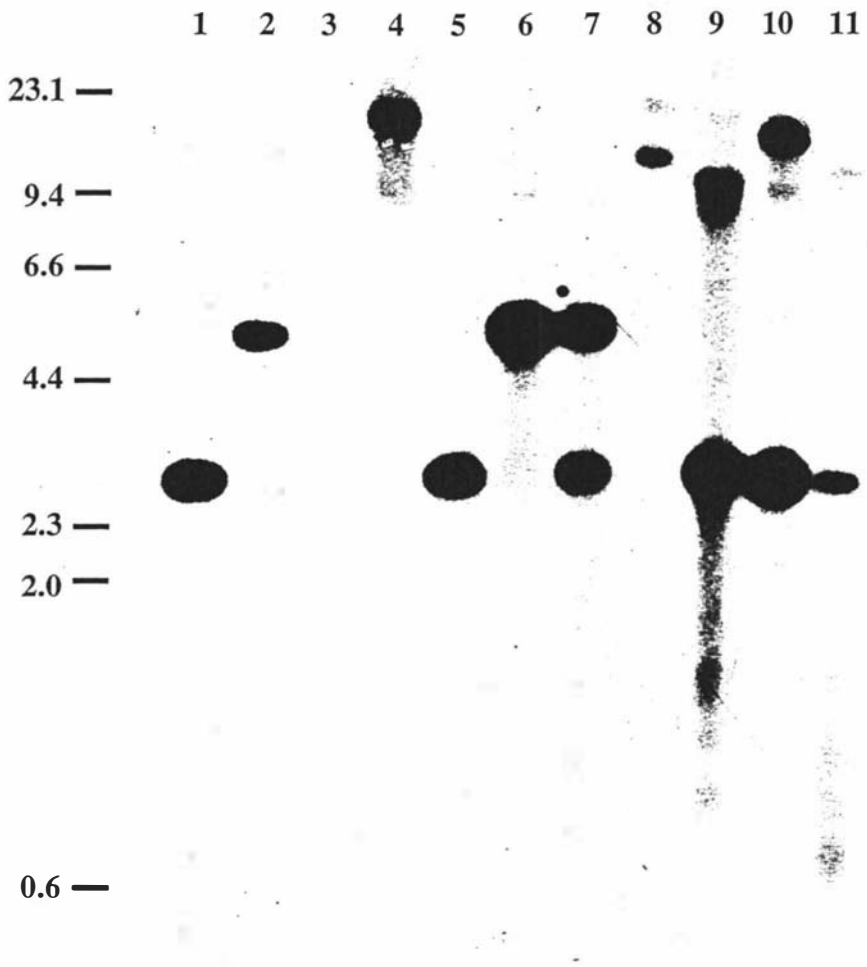

Fig. 22. Polymorphisms of *pyr*4 genes in a range of *Acremonium* and *Epichloë* species.

Autoradiograph of a Southern blot of *Bam*HI digested total DNA from λMC11 (lane 1), λMC12 (lane 2), *Epichloë festucae* isolate (= MP-II) E32 (lane 4), *A. lolii* (= LpTG-1) isolate Lp5 (lane 5), *E. typhina* isolate (= MP-I) E8 (lane 6), LpTG-2 isolate Lp1 (lane 7), *A. uncinatum* isolate Tf4 (lane 8), FaTG-2 isolate Tf15 (lane 9), FaTG-3 isolate Tf18 (lane 10) and *A. coenophialum* (= FaTG-1) isolate Tf28 (lane 11) hybridised to an [α-^{32}P]dCTP labelled 2.2 kb *Bam*HI-*Eco*RI fragment from pMC2. Numbers on the left of the figure indicate the position and size (kb) of fragments generated by a *Hin*dIII digest of λ DNA.

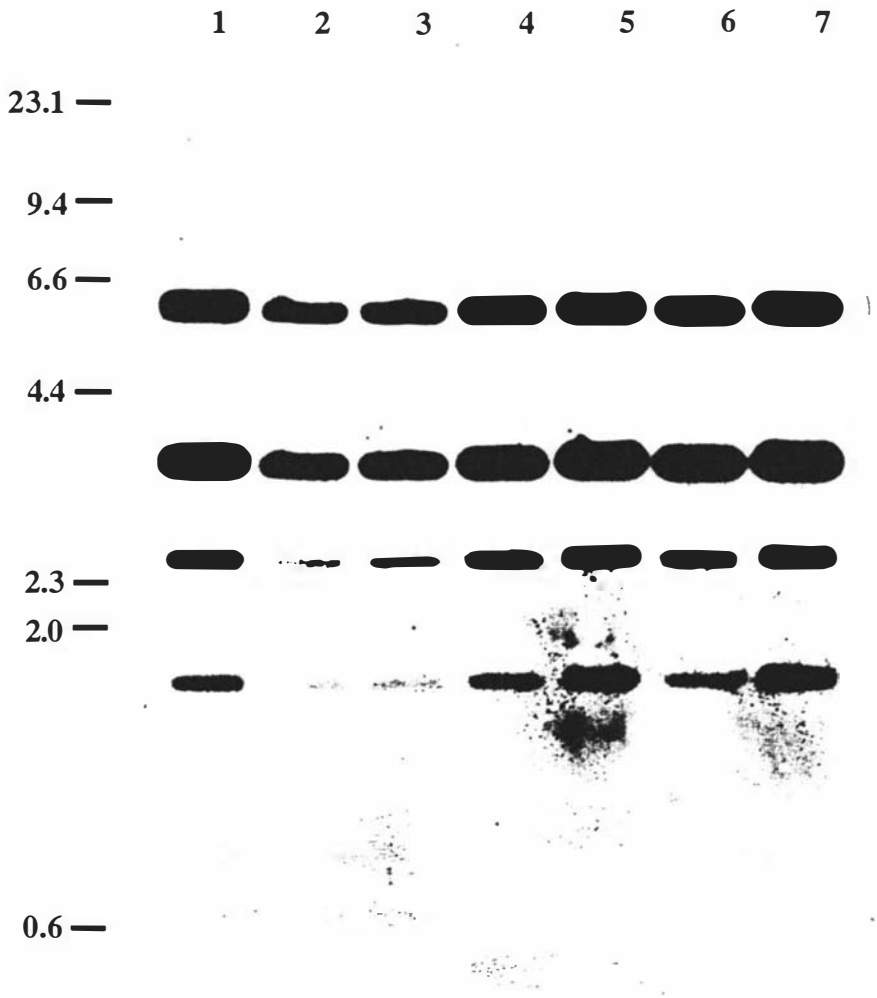

Fig. 23. Demonstration that Lp1 is homokaryotic for *pyr*4-1 and *pyr*4-2.

Autoradiograph of a Southern blot of *Bam*HI digested total DNA from Lp1 (lane 1), Lp1 single spored isolate 1 (ss1, lane 2), ss5 (lane 3), ss10 (lane 4), ss12 (lane 5) ss16 (lane 6), and ss20 (lane 7) hybridised to an [α-^{32}P]dCTP-labelled PCR product from *pyr*4-2 generated with primers mc1 and mc8. Numbers on the left of the figure indicate the position and size (kb) of fragments generated by a *Hin*dIII digest of λ DNA.

3.6.3 Sequence Comparisons of the 5' Non-Coding Sequences of *pyr*4 from *Acremonium* and *Epichloë* Species

In an attempt to further resolve the ancestry of the two *pyr*4 sequences from strain Lp1 we analysed *pyr*4 sequences from *E.typhina* isolate E8, *E. festucae* isolates E28 and E32, and *A. lolii* isolates Lp5, Lp7, Lp9 and Lp19 (Table 1). The most variable region identified from Lp1 *pyr*4 sequences (ie. the 5' non-coding sequences) was chosen for analysis. This region was amplified by PCR (Section 2.15) from total DNA of these isolates using primers mc4 and mc8 (Figs. 10 and 12), directly sequenced from PCR products (Section 2.16), and nucleotide sequences manually aligned with corresponding regions of Lp1 *pyr*4-1 and *pyr*4-2 sequences using the LINEUP program of GCG (Fig. 24). These isolates were chosen as their evolutionary relationships have already been explored by a combination of *pyr*4 RFLPs, phylogenetic analysis of *tub*2 and *rrn* nucleotide sequences, and analysis of isozymes, morphological characteristics and alkaloid production in fungal-host associations (Christensen *et al.* 1993, Figs 20-22; Schardl *et al.* 1994). These investigations had identified *E. typhina* isolate E8 and isolates from the *A. lolii* grouping (Christensen *et al.* 1993) as the most likely ancestors of the Lp1 interspecific hybrid (Schardl *et al.* 1994). *A. lolii* isolates were chosen that included four of the six isozyme phenotypes identified by Christensen *et al.* (1993). Inspection of aligned sequences reveals that 5' non-coding sequences of individual Lp1 *pyr*4 genes are much more closely related to 5' non-coding sequences of proposed ancestors of Lp1 (Schardl *et al.* 1994) than to each other (Fig. 24). *pyr*4-1 and *pyr*4-2 5' non-coding sequences differ from each other at a total of 53 sites (counting indels and differences immediately adjacent to indels as only one difference) but Lp1 *pyr*4-1 5' non-coding sequence and 5' non-coding sequences of *pyr*4 from *E. typhina* isolate E8 are nearly identical having just one difference, while 5' non-coding sequences of *pyr*4-2 from Lp1, Lp5, Lp7, Lp9, Lp19, E28 and E32 are also almost identical. Lp1 *pyr*4-2 sequence differs from Lp5, Lp7, Lp9, Lp19 and E28 sequences in only two positions. 5' non-coding sequence of *E. festucae* isolate E32 differs from Lp1 *pyr*4-2 sequence in one additional position. Of the three sites which vary between 5' non-coding sequences of *pyr*4-2 from Lp1, Lp5 and E32, two sites are identical between Lp5 and E32 while differing from corresponding Lp1 sites and one site is identical between Lp1 and Lp5 but different from E32. These results therefore confirm the previously proposed ancestry of *Acremonium* isolate Lp1. In summary, the two *pyr*4 sequences from Lp1 are clearly very distinct, and obviously more closely related to *pyr*4 sequences of other *Acremonium* and *Epichloë* spp. than they are to each other. This lends further support to the hypothesis that the presence of these two genes within Lp1 occurred by interspecific hybridisation as previously suggested (Schardl *et al.* 1994) and agrees with the phylogeny presented

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                .         .         .         .         .         .
pyr4-1  GTCTAAGGAAAGGGTAATTATTA----------CATGTAGGGATTAAGACCCCTTTTCTT   50
E8      GTCTAAGGAAAGGGTAATTATTA----------CATGTAGGGATTAAGACCCCTTTTCTT   50
pyr4-2  GTCTAAGGAAAGTGGAATTATTAGGGATTATGTTATCAAGGG-TTAAACCCCCTTTT--T   57
Lp5     GTCTAAGGAAAGTGGAATTATTAGGGATTATGTTATCAAGGG-TTAAACCCCCTTTT--T   57
E32     GTCTAAGGAAAGTGGAATTATTAGGGATTATGTTATCAAGGG-TTAAACCCCCTTTT--T   57
                .         .         .         .         .         .
pyr4-1  ACTATCAGAGGTCTTATTAATA----TGGATAACTTACTTAATCTGTC----CAGGGCGA  102
E8      ACTATCAGAGGTCTTATTAATA----TGGATAACTTACTTAATCTGTC----CAGGGCGA  102
pyr4-2  ACTATTAGAGGGCTTATTAATACATATGGATAACTTACTCAATCTGTCTATGTAAGGCGA  117
Lp5     ACTATTAGAGGGCTTATTAATACATATGGATAACTTACTCAATCTGTCTATGTAAGGCGA  117
E32     ACTATTAGAGGGCTTATTAATACATATGGATAACTTACTCAATCTGTCTATGTAAGGCGA  117
                .         .         .         .         .         .
pyr4-1  TTATCCCGTA---ATTGGTGGACGCGAAGGGGGGAAGCTGTGCCCCACTCCCCTTACGAA  159
E8      TTATCCCGTA---ATTGGTGGACGCGAAGGGGGGAAGCTGTGCCCCACTCCCCTTACGAA  159
pyr4-2  CTATCCTGTATGTAGTGGTAAACGCGAAGGGGGGGAGGTGGACCCCACTCCCCTTATGAA  177
Lp5     CTATCCTGTATGTAGTGGTAAACGCGAAGGGGGGGAGGTGGACCCCACTCCCCTTATGAA  177
E32     CTATCCTGTATGTAGTGGTAAACGCGAAGGGGGGGAGGTGGGCCCCACTCCCCTTATGAA  177
                .         .         .         .         .         .
pyr4-1  AATGTTTCTCCGCAGGAGATAGTTGGGGGTTTTTTTTTTCTTCAT--CCTTATT-----AA  212
E8      AATGTTTCTCCGCAGGAGATAGTTGGGGGTTTTTTTTTTCTTCAT--CCTTATT-----AA  212
pyr4-2  AATGTTTCTCCGCAGG-----------TTTTTTTTTTTCTTCTCTACCTCATAATAAGAA  226
Lp5     AATGTTTCTCCGCAGG------------TTTTTTTTTTCTTCTCTACCTCATAATAAGAA  225
E32     AATGTTTCTCCGCAGG------------TTTTTTTTTTCTTCTCTACCTCATAATAAGAA  225
                .         .         .         .         .         .
pyr4-1  GCTAATTGACTTTTGTTCATGAAAACATCATCTGAGTATTGACCCTACATTGTTTCACCG  272
E8      GCTAATTGACTTTTGTTCATGAAAACATCATCTGAGTATTGACCCTACATTGTTTCACCG  272
pyr4-2  GCTAATTGACTTTTGTTTATGAAACCATCATCTGGGTATTGACCCTGCATTGTTTCATCG  286
Lp5     GCTAATTGACTTTTGTTTATGAAACCATCATCTGGGTATTGACCCTGCATTGTTTCATCG  285
E32     GCTAATTGACTTTTGTTTATGAAACCATCATCTGGGTATTGACCCTGCATTGTTTCATCG  285
                .         .         .         .         .         .
pyr4-1  TCACATTTTTTTGCCGAGGAACTTGTCTACTTGCGCAAATTCAATATATCTTGACAAAGC  332
E8      TCACATTTTTTTGCCGAGGAACTTGTCTACTTGCGCAAATTCAATATATCTTGACAAAGC  332
pyr4-2  TCAAATTTTATTGTCAAGAAACTTATTGTCTTGCACAAATTCAGTATATATTGACAAAGC  346
Lp5     TCAAATTTTATTGTCAAGAAACTTATTGTCTTGCGCAAATTCAGTATATATTGACAAAGC  345
E32     TCAAATTTTATTGTCAAGAAACTTATTGTCTTGCGCAAATTCAGTATATATTGACAAAGC  345
                .         .
pyr4-1  CTTTTACGTACCAAACGATCCAAATATT  360
E8      CTTTTACGTACCAAACGATCCAAATATT  360
pyr4-2  CTTTTACCCACCAAAAGATTCAAATATT  374
Lp5     CTTTTACCCACCAAAAGATTCAAATATT  373
E32     CTTTTACCCACCAAAAGATTCAAATATT  373
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Fig. 24. Sequence alignment demonstrating that *pyr*4-1 and *pyr*4-2 sequences are more similar to sequences from *Epichloë typhina* and *Acremonium lolii* than to each other.

Alignment of 5' non-coding nucleotide sequences from *pyr*4 of various *Acremonium* and *Epichloë* isolates. Sequences for E28, Lp7, Lp9 and Lp19 were identical to that of Lp5 and therefore are not shown. Variant sites are in bold. Alignment gaps are indicated by dashes (-). Alignments were performed manually. Sequences were deposited in the GenBank sequence database under accession numbers U14561 (E32), U14562 (E8) and U14563 (Lp5). As E28, Lp7, Lp9 and Lp19 sequences were identical to Lp5 these sequences were not submitted.

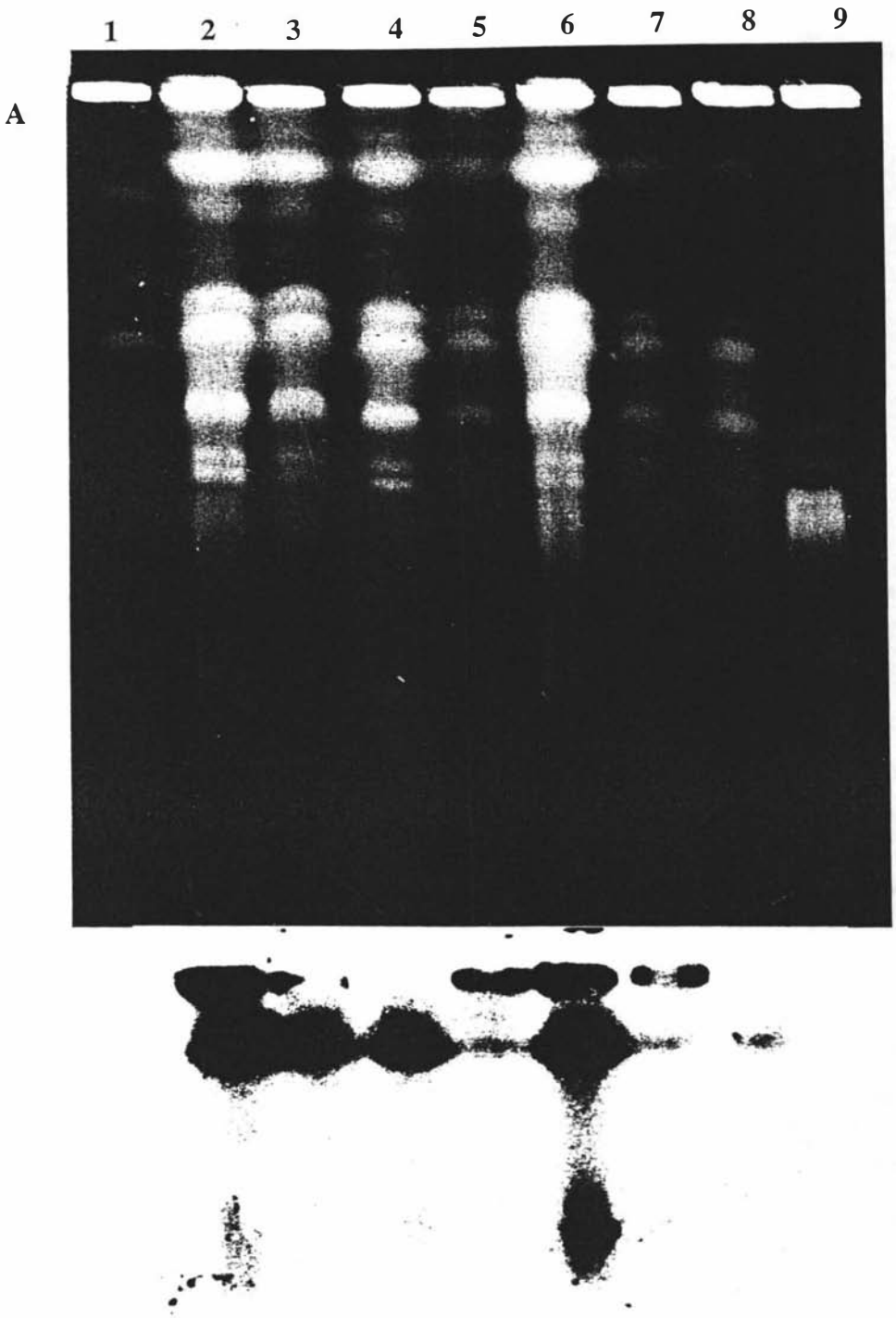
therein. Given the similarity of *pyr*4 5' non-coding sequences from Lp1 to that of the proposed ancestors it seems likely that the interspecific hybridisation event which gave rise to Lp1 is a relatively recent event and there has been little divergence of the nucleotide sequences since this event.

3.7 CHEF GEL ANALYSIS OF THE CHROMOSOMAL LOCATION OF *PYR*4-1 AND *PYR*4-2

Using conditions to resolve chromosomal DNA of Lp1 by clamped homogenous electric field (CHEF) gel electrophoresis given in Section 2.23, eleven chromosomes were resolved from Lp1 and six single spored isolates on a 0.6% chromosomal grade agarose gel (Fig. 25A). A Southern blot of this gel was hybridised (Section 2.12) to a PCR product from *pyr*4-1 generated with primers mc1 and mc5 (Section 2.15). This demonstrated that the two *pyr*4 genes were in a hybridising band corresponding to chromosome X identified by Murray *et al.* (1992). This band appears to be a doublet when one compares the intensity of ethidium bromide staining of this band to other similar sized chromosomal bands (Fig. 25A). Thus *pyr*4-1 and *pyr*4-2 may be on one chromosome of about 6.0 Mb, or two separate chromosomes of a similar size.

ig. 25A-B. CHEF gel showing chromosomal position of *pyr*4-1 and *yr*4-2 and that Lp1 is a homokaryon.

Chromosomal DNA of *Schizosaccharomyces pombe* (lane 1), Lp1 (lane 2), Lp1 ngle spored isolate 1 (ss1, lane 3), ss5 (lane 4), ss10 (lane 5), ss12 (lane 6), ss16 (lane), ss20 (lane 8) and *Saccharomyces cerevisiae* (lane 9) separated by contour-clamped omogenous electric field (CHEF) gel electrophoresis. **B** Autoradiograph of a Southern ot of gel shown in A hybridised to a PCR product from *pyr*4-2 generated with primers c1 and mc8.


1
2
3
4
5
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9
A

Chapter 4.0 DISCUSSION

4.1 IMPLICATIONS OF DUPLICATE *PYR*4 IN LP1

An endophyte isolated from perennial ryegrass, designated Lp1 (Christensen *et al.* 1993), which is currently classified as an *Acremonium* sp. to which a specific epithet is yet to be given, but placed in taxon *L. perenne* taxonomic group two or LpTG-2 (Christensen *et al.* 1993), was found to possess two sequences hybridising to *C. purpurea pyr*4 (Section 3.2.3). These regions were cloned (Section 3.2), and sequencing of the cloned regions revealed two very similar nucleotide sequences (95.4% identity). Both of these sequences showed similarity to OMPdecarboxylase genes from other filamentous fungi, and possessed features that were common to OMPdecarboxylase genes from Pyrenomycetes (Section 3.3). Transcripts from both genes were present in total RNA from Lp1 (Section 3.4), and both genes were able to complement a mutation in the OMPdecarboxylase gene of an *A. nidulans* strain (Section 3.5). Thus both of these sequences encode functional OMPdecarboxylases, hence they were designated *pyr*4-1 and *pyr*4-2. On a Southern blot of Lp1 chromosomal DNA separated by CHEF gel electrophoresis, *pyr*4-1 was shown to hybridise to a single band of very high molecular weight, under hybridisation conditions which would detect both *pyr*4. This band most probably corresponds to a doublet, suggesting that the two *pyr*4 are either on two different chromosomes, or just one (Section 3.7).

Other *Acremonium* and *Epichloë* isolates were studied for *pyr*4 RFLPs, in an attempt to determine the relationships of *pyr*4-1 and *pyr*4-2 from Lp1 to *pyr*4 in these isolates (Section 3.6). Another endophyte from the taxonomic group LpTG-2, isolate Lp2, was shown to have RFLPs identical to Lp1, suggesting that Lp2 possesses two *pyr*4 similar to those of Lp1. In addition *A. lolii* (= LpTG-1) isolates Lp5, Lp6 and Lp9, *A. uncinatum* (= FpTG-1) isolate Tf4, *A. coenophialum* (= FaTG-1) isolate Tf28, FaTG-2 isolate Tf15, FaTG-3 isolate Tf18, *E. festucae* isolate E32, and *E. typhina* isolate E8 were analysed for *pyr*4 RFLPs. This revealed that isolates of *A. lolii* (an endophyte of perennial ryegrass) and *E. typhina* (the ryegrass choke pathogen) each possessed one *pyr*4 copy. The combined RFLPs for these were identical to the RFLPs observed for LpTG-2 isolates. This suggested that the two *pyr*4 in Lp1 had separate evolutionary origins; *pyr*4-1 originating from *E. typhina* and *pyr*4-2 from *A. lolii* (or close relatives of these, see Section 3.6 for experimental evidence). The two *pyr*4 in Lp1 were shown to be in the same nucleus (Section 3.6.2), suggesting that Lp1 is a homokaryon.

Two similar mechanisms for the separate evolutionary origins of *pyr*4-1 and *pyr*4-2 in Lp1 are proposed: either horizontal transfer of a small amount of DNA

containing a *pyr*4 from one ancestor into another giving rise to Lp1 (e.g. by viral transduction or B-chromosome transfer); or interspecific hybridisation (a form of horizontal transfer) of the two ancestors giving rise to Lp1. Analysis of DNA polymorphisms, nucleotide sequences and isozymes in Lp1 (Schardl *et al.* 1994) revealed that Lp1 also possessed duplicated copies of *tub*2 (β-tubulin), aconitase, aldolase, leucine aminopeptidase, malate dehydrogenase, phosphoglucose isomerase and phosphoglucomutase-1 and -2. These results support the hypothesis that interspecific hybridisation was the mechanism by which the duplicate gene copies arose. Furthermore, a comparison of the *pyr*4 5' non-coding nucleotide sequences from Lp1, Lp5, Lp7, Lp9, Lp19, E28, E32 and E8 demonstrated that: (i) *pyr*4-1 from Lp1 was very similar to the E8 *pyr*4 (1 difference), but differed at many sites from the Lp1 *pyr*4-2 (53 differences); and (ii) *pyr*4-2 from Lp1 was very similar to the *pyr*4 sequences from Lp5, Lp7, Lp9, Lp19, E28 and E32 (2 or 3 differences, Section 3.6.3). Schardl *et al.* (1994) compared the nucleotide sequences of *tub*2, mitochondrial DNA RFLPs, isozyme polymorphisms and *pyr*4 RFLPs and identified E8 and *A. lolii* isolates as being the most likely ancestors of Lp1. In particular, *A. lolii* isolate Lp5 and *E. typhina* isolate E8 were identified as being the most likely ancestors of Lp1. The *E. festucae* endophytes (close relatives of *A. lolii* based on *tub*2, *pyr*4 and rDNA nucleotide sequences) were very similar to half of the Lp1 genotype, but less so than Lp5. Thus, it seems likely that Lp1 arose by interspecific hybridisation between the ryegrass choke pathogen, *E. typhina*, and an asexual endophyte from perennial ryegrass, *A. lolii*. This presumably occurred by *E. typhina* infection of a plant already host to an *A. lolii*, and subsequent hyphal fusion (anastomosis) of the two isolates, followed by fusion of the two different nuclei (karyogamy). Subsequently, mitotic recombination may have occurred between the two genomes, and genetic material may have been lost, in a process similar to the parasexual cycle, but without a return to the haploid state. That recombination or loss of genetic material subsequent to the hybridisation event has occurred in Lp1 seems certain, as only one sequence from the ITS1, 5.8S and ITS2 region of the rDNA cluster was detected in Lp1 (Schardl *et al.* 1994) . This could be the result of deletion of one rDNA copy, or gene conversion resulting in homogeneity of these sequences. In addition, the NTS region of the rDNA from Lp1 shows extraordinary length heterogeneity, and this heterogeneity changes with mitotic growth (A. Ganley, unpublished data). Thus, the genome of Lp1 is still undergoing change, at least in the NTS region of rDNA.

The similarities of the *pyr*4 5' non-coding nucleotide sequences from *E. festucae* and *A. lolii* suggest that these two species are closely related. This is supported by *tub*2 and rDNA sequences as well as *pyr*4 and isozyme polymorphisms (Schardl *et al.* 1994). Mating tests (C. Schardl, pers. comm.) have shown that *A. lolii* isolates are unable to complete the sexual cycle when crossed with *E. festucae*. This indicates that *E.*

festucae and *A. lolii* are reproductively isolated and are therefore distinct species. Thus, it seems likely that *A. lolii* has evolved from *E. festucae*, and in the process has lost it's sexual cycle. Two similar possible scenarios are proposed for these events (Schardl *et al.* 1994). An *E. festucae* (from it's normal host, a *Festuca* sp.) presumably infected perennial ryegrass which did not contain an endophyte. Subsequent to this infection the fungus either lost it's sexual stage (stromatal expression) through mutation, or the new host grass suppressed the stromatal expression of the fungus. This fungus would have then given rise to *A. lolii*.

In fungi, interspecific hybridisation is likely to occur by one of two possible mechanisms: either an interspecific sexual cross; or interspecific hyphal anastomosis. It is quite conceivable that an *E. festucae* isolate (which probably gave rise to *A. lolii* by loss of the sexual cycle as outlined above), not sampled in these investigations, possesses a genotype similar to Lp5, and gave rise to Lp1 by a rare interspecific cross between *E. typhina* and this *E. festucae*. However, if an *A. lolii* (e.g. Lp5) is an ancestor of Lp1, then the individuals giving rise to Lp1 were members of the same vegetative compatibility group (VCG). Vegetative incompatibility is generally thought to be widespread (perhaps universal) within fungal populations (Glass and Kuldau 1992; Leslie 1993) and has been studied intensely in a number of fungal species (e.g. *Aspergillus*, *Fusarium*, *Neurospora* and *Podospora*). It is typically controlled by multiple loci, hence isolates which are members of the same VCG are usually genetically extremely similar (Leslie 1993). As interspecific genetic variation is, as a rule, greater than intraspecific genetic variation, isolates of *E. typhina* and *A. lolii* as members of the same VCG would set an important precedent for studies of fungal vegetative compatibility. No hyphal incompatibility was observed when interspecific or intraspecific sexual crosses of *Epichloë* spp. were attempted, suggesting that *Epichloë* spp. are all members of the same VCG (Schardl *et al.* 1994). However, in many fungi, sexual and vegetative compatibility is quite distinct (Leslie 1993), so this observation is not proof of a lack of multiple VCGs in *Epichloë*. Further evidence that isolates from separate *Epichloë* and *Acremonium* spp. are in identical VCGs, is the occurrence of *A. coenophialum* endophytes of *Festuca arundinacea* that possess three *tub*2 copies as a result of interspecific hybridisation (Tsai *et al.* 1994). This observation suggests that at least two hybridisation events occurred during the evolution of *A. coenophialum*; the first hybridisation event either by an aberrant interspecific sexual cross, or hyphal fusion by members of the same VCG. After this event the hybrid may have been asexual, and vegetative compatibility would have been necessary for the second hybridisation event to occur (Tsai *et al.* 1994). However, it is possible that the hybrid was sexually fertile, and then underwent another aberrant cross. Whatever the case, the presence or absence of VCGs between isolates of *Acremonium* and *Epichloë* spp. is testable (Leslie 1993).

Endophytes from *F. arundinacea* taxonomic groups FaTG-1, FaTG-2 and FaTG-3 all possessed a *pyr*4 RFLP similar to that exhibited for *pyr*4-2, but they also possessed additional RFLPs (Section 3.6.1). FaTG-1 and FaTG-2 possess the *tub*2 gene found in *A. lolii* (Tsai *et al.* 1994, *tub*2-2), and so by inference may also possess *pyr*4-2. Although FaTG-3 lacks *tub*2-2, it may still possess *pyr*4-2, as *F. arundinacea* endophytes are thought to have lost genes after hybridisation events (Tsai *et al.* 1994). Conversely, the *pyr*4 RFLPs could correspond to another *pyr*4 that has not been characterised. Whatever the case, as Lp1 shares many similarities with *A. lolii*, it seems unlikely that either FaTG-1, FaTG-2 or FaTG-3 would be one of the direct ancestors of Lp1.

Comparisons of nucleotide sequences of the *pyr*4 region could well be of use in further resolving the ancestry of the *F. arundinacea* endophytes, as the evolutionary scenario proposed for the origins of multiple *tub*2 copies in these endophytes is complex, and other scenarios are possible (Tsai *et al.* 1994). In addition, isozyme polymorphisms of asexual endophytes and sexual *Epichloë* spp. have shown that many other asexual endophytes possess gene duplications, suggesting that interspecific hybridisation between *Epichloë* and endophytes may be common (Christensen *et al.* 1993; Leuchtmann 1994; Leuchtmann and Clay 1990). Again, comparisons of *pyr*4 nucleotide sequences could be of use in resolving the ancestry of these endophytes.

Lager yeasts (e.g. *Saccharomyces carlsbergensis* lager yeast M204 or strain 244) have also been shown to be allopolyploid interspecific hybrids, most likely between *S. cerevisiae* and either *S. bayanus* or *S. monacensis* (Casey 1990). Analysis of two *MET*2 genes from *S. carlsbergensis* strain 244 revealed one *MET*2 with a nucleotide sequence identical, or very similar, to *MET*2 of *S. cerevisiae*, and the other *MET*2 had a nucleotide sequence identical to 330 bp of PCR amplified *MET*2 from *S. monacensis*, but only 93% identical to the *S. bayanus* sequence. This suggested that this strain was an interspecific hybrid between *S. cerevisiae* and *S. monacensis* (Hansen and Kielland-Brandt 1994). This hybrid most probably arose by an aberrant chance interspecific sexual cross between isolates of these two species. This well-documented case indicates that interspecific hybridisation in the Ascomycetes is not limited to endophytes and *Epichloë*. Interspecific hybridisation of plant pathogens may have important implications for plant pathology. The fungal pathogen responsible for Dutch elm disease, *Ophiostoma novo-ulmi*, may also have arisen by interspecific hybridisation (Brasier 1994). Interspecific hybridisation may be a mechanism whereby new species of fungi, which have previously unseen pathogenic capabilities (much like *O. novo-ulmi* and *O. ulmi* on elms), may rapidly evolve.

Interspecific hybridisation is also common in higher eukaryotes. It is considered to be an important evolutionary phenomenon in plants (Grant 1981). The importance of it's role in animal evolution is debatable, some authors consider interspecific hybridisation not to be of importance (Mayr 1963), but the recent finding of a number of hybrid animal species has lead to calls for a re-evaluation of it's importance (Bullini 1994). Interspecific hybridisation could well have been fundamental in the evolution of higher animals from their lower evolutionary ancestors (Ohno 1970). The evolutionary importance of interspecific hybridisation in fungi is unknown at this stage, and awaits further investigation. The point should be made, however, that in all the *Acremonium* interspecific hybrids identified to date, for which the ITS sequence (of the rDNA region) has been determined, only one ITS sequence has been identified (Schardl *et al.* 1994; Tsai *et al.* 1994). The ITS sequences had previously failed to identify these isolates as interspecific hybrids (An *et al.* 1992; Schardl *et al.* 1991; Schardl and Tsai 1992). In addition, investigations of mitochondrial DNA (mtDNA) in Lp1, revealed that Lp1 possessed mtDNA polymorphisms found in only one of the proposed ancestors (Schardl *et al.* 1994). The biological basis for these observations is unknown, but highlights the limitations of using only one genetic marker, particularly rDNA or mtDNA, for determining phylogenies of asexual fungi.

The manner in which most asexual fungi are thought to have evolved from their sexual ancestors is by loss of function mutations in genes involved in the sexual cycle (LoBuglio *et al.* 1993). The resulting imperfect strains are meiotically sterile, and limited to asexual (mitotic) reproduction. The identification of asexual *Acremonium* interspecific hybrids demonstrates that interspecific hybridisation is another mechanism by which asexual fungi in particular may have evolved from their sexual relatives. The loss of the sexual cycle in interspecific hybrids is presumably a result of chromosomal sterility; that is homologous chromosomes either fail to pair normally during meiosis, or the chromosomes pair but segregate to give daughter nuclei carrying deletions and duplications. It should be noted that this sterility can be overcome by doubling the chromosome number, as commonly occurs in plants (Grant 1981), although the chromosome doubling process is incompatible with the chromosomal sex determining mechanism that is present in many animal species (Ohno 1970).

The apparently widespread occurrence of hybrid endophytes suggests hybridisation is important to these organisms (Leuchtmann 1994; Leuchtmann and Clay 1990; Schardl *et al.* 1994; Tsai *et al.* 1994). A number of possible benefits can be proposed. Interspecific hybridisation will result in widespread gene duplication in the endophytes, this is considered to be an important evolutionary event, especially for the evolution of genes with new functions (Ohno 1970). Gene duplications create genetic

redundancy. This allows genes, which already possess a specific function, to evolve new functions, while one copy of the duplicated gene continues to perform the function it was originally selected for, and is constrained by natural selection to perform. In addition, the asexual endophytes may be susceptible to Muller's ratchet (Muller 1964), that is the relentless accumulation of marginally deleterious mutations, causing gradual loss of fitness, in asexual organisms. Meiotic recombination is considered an important means by which these deleterious mutations are removed in organisms possessing a sexual stage in their life cycle. However, asexual organisms lack a means of removing these mutations from their genomes (except for back mutation, which is probably very rare) hence it has been argued that asexual organisms are doomed to eventual extinction as a result of this process. Hybridisation may provide the endophytes with means of avoiding this. In addition, it seems likely that interspecific hybridisation has provided hybrid endophytes with considerable genetic variation (Tsai *et al.* 1994). Multiple copies of divergent genomes provide the asexual endophytes, and their symbiotic plant hosts, with greater genetic diversity than would be found in a genome reproducing in a clonal fashion, and could be of considerable importance to the plant and endophyte, especially in a variable environment. This could, initially, be simply achieved by the plant acting as host to a genetically distinct endophyte and (following infection) an *Epichloë*. If these two isolates were members of the same VCG, or their incompatibility was overcome, then eventual hybridisation of the two is conceivable. The implications of the presence in one plant of an endophyte (e.g. *A. lolii*), which is thought to be solely seed disseminated, and an aggressive choke pathogen (e.g. the perennial ryegrass choke pathogen, *E. typhina* isolate E8), which consistently sterilises developing inflorescences of infected plants, has been considered by Schardl *et al.* (1994). They raised the point that the life-cycle of the choke pathogen is possibly restrictive to the plant and endophyte. A genetic change in the association may be of benefit to the plant. For example hybridisation may be a means by which an aggressive antagonist, such as *E. typhina*, could be disarmed by a related mutualist. Which of these factors, if any, are important to the symbiotic association formed between the endophytes and their grass hosts remains to be determined. Measuring the fitness and frequency of naturally occurring associations of grasses and hybrid endophytes, as compared to the fitness and frequency of associations involving non-hybrid endophytes, under a wide range of environmental conditions, may give useful data as to the importance of the increased genetic variation and other advantages or disadvantages possessed by the hybrids. If hybridisation is indeed a means for the endophytes to overcome Muller's ratchet, then a prediction from this would be that asexual hybrid endophyte lineages would tend to be more ancient than asexual non-hybrid endophyte lineages. A means of testing this would require identifying genes present in the endophyte since the initial hybridisation event occurred, and identification of the nearest living sexual and asexual non-hybrids, and comparing the divergence of

these sequences. Assuming a constant molecular clock between the sequences studied, one would expect the hybrids to be a greater genetic distance from their ancestral sexual species, than the non-hybrids.

Several lines of evidence suggest that the hybridisation event giving rise to Lp1 was quite recent. These include the similarities of the Lp1 5' non-coding nucleotide sequences from *pyr*4-1 and *pyr*4-2 to the analogous sequences from *E. typhina*, *E. festucae* and *A. lolii* isolates (Section 3.6.3). The fact that both *pyr*4 genes are functional and expressed at similar levels (Sections 3.4.1 and 3.5), that *tub*2-1 and *tub*2-2 sequences from Lp1 are almost identical to the same regions from E8 and Lp5, and that E8 and Lp1 share identical ITS sequences (Schardl *et al.* 1994). Conversely, the *tub*2-4 sequences of the hybrid endophytes identified from *F. arundinacea* by Tsai *et al.* (1994), in particular, are quite distinct from the *tub*2 sequence of the nearest known *Epichloë* relative. This sequence is thought by Tsai *et al.* (1994) to be the *tub*2 sequence introduced in the first hybridisation event giving rise to multiple *tub*2 in these endophytes. Thus this hybridisation event may be an ancient one relative to the event which gave rise to Lp1.

As hybrids appear to be common in the endophytes, it may be relatively easy to generate new strains of endophytes, with useful agronomic properties, by hybridising endophytes, which possess desirable agronomic traits. The ability to generate a hybrid endophyte *in planta* is currently being tested (M. R. Siegel and C. L. Schardl, pers. comm.). Any new strains so generated would require rigorous testing as to their stability and agronomic usefulness.

4.2 SEQUENCE FEATURES OF OROTIDINE-5'-MONOPHOSPHATE DECARBOXYLASES

A fungal phylogeny, based upon the amino acid sequences of OMPdecarboxylase has been published recently (Radford 1993). The relevant portion of this phylogeny compares well to the phylogeny for the filamentous members of the Ascomycetes given in this thesis (Section 3.3.5.2), with these fungi forming two main clades: one containing the Pyrenomycetes and related Deuteromycetes, including the two similar Lp1 OMPdecarboxylase sequences (OMPD-1 and OMPD-2); and the other containing the Plectomycetes and related Deuteromycetes. Other members of the filamentous Ascomycetes were not included in either of these analyses, due to a lack of sequences. The placing of OMPD-1 and OMPD-2, from Lp1, with the Pyrenomycetes is as expected, as the *Acremonium* endophytes (which according to traditional taxonomy are classified as Deuteromycetes) are thought to have evolved from *Epichloë* (Schardl *et al.* 1991), a Pyrenomycete. The gene sequences of OMPD-1 and OMPD-2 both possess

structural features common to OMPdecarboxylases from other Pyrenomycetes (Section 3.3.5), supporting the placement of OMPD-1 and OMPD-2 with other Pyrenomycetes.

Comparisons of OMPdecarboxylase genes reveal some interesting trends. Intron position, and other structural gene features, have long been regarded as a useful indicator of phylogeny, although, as with all evolutionary data, on their own these characters can be misleading. In the OMPdecarboxylase genes from the Pyrenomycetes and related Deuteromycetes, a universal feature is a large insert in the middle of all the sequences determined to date, as well as a lack of introns. The other Ascomycetes that have been examined, the Plectomycetes and their related Deuteromycetes, lack this insert, and possess an intron in the N-terminus of the gene, which is conserved in position (Radford 1993). Other structural features of the OMPdecarboxylase genes are given in Radford (1993).

4.3 POTENTIAL USES OF *PYR*4

OMPdecarboxylase encoding genes, and strains containing mutants of this gene, have been widely used in transformation systems in fungi. There are powerful selection systems available for loss of function mutants of OMPdecarboxylase in yeast, and these systems have proven useful in other fungi (Boeke *et al.* 1984). These mutants are relatively easily selected with 5-fluoro-orotic acid (positive selection) and require uridine or uracil for growth (negative selection).

Given the *pyr*4-1 and *pyr*4-2 clones now available from Lp1 (Table 1), it should be possible to perform gene disruption experiments at the *pyr*4 loci of *Acremonium* and *Epichloë* spp. This was the initial intention of the work undertaken in this thesis (Section 1.4), however during the course of this work it was decided to investigate the aspects of the *pyr*4 genes in Lp1 outlined herein. It is desirable to perform gene disruptions in strains which do not possess gene duplications (i.e. non-hybrids, such as *A. lolii* isolates). It may be possible, using PCR, to isolate fragments of *pyr*4 from *Acremonium* or *Epichloë* isolates, and use these fragments to synthesise constructs for disruption of this gene, if the size of these fragments is sufficient for homologous recombination to occur between the transforming DNA and the resident *pyr*4. Conversely, the *pyr*4 clones generated in this work (Table 1), or appropriate PCR products, should be suitable to use as probes for isolating *pyr*4 clones from related isolates, assuming suitable gene libraries are available. Given the conservation of the variable 5' non-coding regions between *pyr*4-1 and the *E. typhina pyr*4 sequence, and *pyr*4-2 and the *A. lolii* and *E. festucae pyr*4 sequences (Section 3.6.3), it seems likely that the structural regions of these genes will also be highly conserved. Hence, cloned *pyr*4-1 and *pyr*4-2 may be useful for performing gene disruptions in the appropriate *E.*

typhina, *E. festucae* and *A. lolii* isolates, and other strains possessing *pyr*4-1 or *pyr*4-2. This has probably already been achieved (C. Berkahn, unpublished data). Clone pMC1 (Table 1) had a cassette containing the hygromycin resistance gene (*hph*) from *E. coli*, under the control of fungal regulatory sequences, inserted into the polycloning site, adjacent to the internal 0.8 kb fragment from *pyr*4-2. This construct was used to transform *A. lolii* isolate Lp19 to Hyg^R and the transformants were screened for single cross-overs by PCR on transformant DNA, using primers inside pUC118 and outside the 0.8 kb *pyr*4-2 fragment in pMC1. Any Hyg^R colonies possessing a single cross-over would possess two truncated *pyr*4 genes, one lacking the 3' sequences and one lacking the 5' sequences, flanking plasmid DNA. These preliminary results suggest that 1 of 12 Hyg^R transformants generated contained a disrupted *pyr*4, although this is yet to be confirmed by a detailed analysis.

Many experimental strategies involving OMPdecarboxylase genes require a strain lacking a functional OMPdecarboxylase. For example the pop-in/pop-out gene replacement method requires a strain lacking a functional OMPdecarboxylase (Scherer and Davis 1979). In endophytes this may be a problem as endophytes auxotrophic for uracil may be unable to grow in the host grass, or may have impaired growth therein. It would be of use to be able to regenerate the wild-type *pyr*4 phenotype in such a strain. This may be achieved in a *pyr*4 mutant of the type generated by Berkahn, and mentioned above. Mutants of this sort, with two truncated genes separated by plasmid DNA, are often unstable, with the plasmid prone to popping-out when selective pressure (Hyg^R in this case) is relaxed, resulting in restoration of the wild-type phenotype. Thus it should be possible to restore the wild type phenotype in a mutant constructed in this fashion, and these can be selected on media lacking uracil or uridine, which will only allow growth of strains containing plasmid pop-outs. For many methods making use of cloned OMPdecarboxylase genes, and strains harbouring mutants of these, it would not be necessary to use constructs containing an OMPdecarboxylase gene cloned from an endophyte, but merely one that is able to efficiently complement an OMPdecarboxylase mutation in an endophyte. This would allow the use of any construct containing OMPdecarboxylase genes expressed in endophytes.

Chapter 5.0 SUMMARY AND CONCLUSIONS

Two *pyr*4 genes (encoding OMPdecarboxylase) were isolated from a genomic library to Lp1, an *Acremonium* endophyte isolated from *Lolium perenne* (currently classified in taxon LpTG-2). These two genes were designated *pyr*4-1 and *pyr*4-2. Nucleotide sequences of *pyr*4-1 and *pyr*4-2 revealed two very similar sequences (95.4% identity). The ORFs were the most conserved portions of the two nucleotide sequences (97.5% identity). The 5' non-coding nucleotide sequences were the most variable regions sequenced (88.5% identity). The 3' non-coding nucleotide sequences had 93.0% identity. The deduced protein sequences obtained by translation of *pyr*4-1 and *pyr*4-2 showed homology to OMPdecarboxylase amino acid sequences, with the strongest homology being to sequences from the Pyrenomycetes and related Deuteromycetes. Comparisons of these protein sequences showed that both genes lacked introns and possessed a large insert (approximately 100 amino acids.) in the middle of the Lp1 OMPdecarboxylase sequences. A lack of introns and the presence of this insert are features which are characteristic of OMPdecarboxylase sequences from Pyrenomycetes (Ascomycetes) and related Deuteromycetes. *Acremonium* endophytes are traditionally classified as Deuteromycetes, due to a lack of a known sexual stage. This data supports the hypothesis that *Acremonium* endophytes have evolved from a sexual species by loss of the sexual cycle.

Comparisons of *pyr*4 RFLPs from Lp1 to *pyr*4 RFLPs from other *Acremonium* endophytes and *Epichloë* spp. revealed that the other endophyte which has been placed in taxonomic group LpTG-2 possessed two *pyr*4 with identical RFLPs to Lp1. Isolates of *E. typhina* (the ryegrass choke pathogen) and *A. lolii* (another endophyte from perennial ryegrass) possessed RFLPs which corresponded with *pyr*4-1 and *pyr*4-2 respectively. In combination these RFLPs were identical to those of Lp1. RFLPs of isolates from *E. festucae*, *A. uncinatum* (FpTG-1), *A. coenophialum* (FaTG-1), FaTG-2 and FaTG-3 were inconsistent with these being likely ancestors of Lp1. This data suggested that Lp1 was an interspecific hybrid, between *E. typhina* and *A. lolii*. Furthermore, comparisons of *pyr*4 5' non-coding nucleotide sequences from Lp1, and isolates of *E. typhina*, *E. festucae* and *A. lolii* revealed that: (i) *pyr*4-1 from Lp1 was almost identical to *pyr*4 from *E. typhina* (1 nucleotide difference), while differing markedly from *pyr*4-2 (53 differences, counting indels as only one difference); and (ii) *pyr*4-2 from Lp1 was almost identical to the *pyr*4 sequences from *E. festucae* and *A. lolii* (2 or 3 nucleotide differences). This suggests *E. typhina* and an *A. lolii* or *E. festucae* are likely ancestors of Lp1. This was supported by analysis of rDNA and *tub*2 nucleotide sequences and mitochondrial DNA and isozyme polymorphisms (Schardl *et al.* 1994), which suggested that an *A. lolii* isolate Lp5 and *E. typhina* isolate E8 are likely

ancestors of Lp1. In addition, the similar *pyr*4 sequences from isolates of *A. lolii* and *E. festucae* suggest that these species are closely related. It is proposed that *A. lolii* has evolved from an *E. festucae* which infected perennial ryegrass, and subsequently lost the sexual stage. This agrees with the data from *tub*2 and rDNA nucleotide sequences as well as mtDNA and isozyme polymorphisms (Schardl *et al.* 1994).

RT-PCR and northern analysis revealed that transcripts of about 1.4 kb from *pyr*4-1 and *pyr*4-2 were present in total DNA of Lp1 at similar levels. In addition both *pyr*4 were able to complement a mutation in the OMPdecarboxylase gene of an *Aspergillus nidulans* strain. Thus both *pyr*4 encode functional OMPdecarboxylases.

On a Southern blot of Lp1 chromosomal DNA separated by CHEF gel electrophoresis, *pyr*4-1 hybridised to a single band of very high molecular weight, under conditions which would detect both *pyr*4. Based on the intensity of ethidium bromide staining the hybridising band most probably corresponded to a doublet, hence the two *pyr*4 are either on two similar sized chromosomes, or just one chromosome.

The *pyr*4 gene is a useful genetic marker. Lp1 *pyr*4-2 has been used to disrupt the *pyr*4 gene in *A. lolii* isolate Lp19 (C. Berkahn, unpublished data). This putative *pyr*4$^-$ strain, and other *pyr*4$^-$ strains may be useful for genetic manipulation of the endophyte-plant symbiosis when used in combination with appropriate vectors carrying functional OMPdecarboxylase genes.

Data presented in this thesis indicates that Lp1 is an interspecific hybrid, most likely between *E. typhina* and *A. lolii.* Given that both *pyr*4 genes are expressed at similar levels, and encode functional OMPdecarboxylases, and the similarity between the 5' non-coding nucleotide sequences of Lp1 *pyr*4 to the corresponding sequences from *E. typhina* and *A. lolii*, it seems likely that the interspecific hybridisation event which gave rise to Lp1 is a relatively recent one. This hybridisation event probably occurred either by hyphal anastomosis, or an interspecific sexual cross. Interspecific hybridisation is apparently widespread in endophytes of grasses (Schardl *et al.* 1994; Tsai *et al.* 1994), suggesting it may be important to these organisms. Thus, it is possible that interspecific hybridisation may be a general phenomenon involved in the evolution of asexual Deuteromycetes species from their sexual ancestors, if not also the evolution of new sexual species. The occurrence of an asexual yeast species (Casey 1990; Hansen and Kielland-Brandt 1994) supports this hypothesis. A number of benefits of hybridisation to the endophytes are proposed. These include hybridisation as a means of creating genetic redundancy (which allows evolutionary plasticity) and as a possible means of overcoming Muller's ratchet (which is considered to be detrimental to asexual organisms). In addition, it provides the asexual endophytes with considerable genetic

variation. If hybrid endophytes can be created in the laboratory it may be a means of generating new endophytes with potentially useful characteristics. Thus interspecific hybridisation may be an important phenomenon in the evolution of fungi.

Appendix 1.0 LIBRARY CONSTRUCTION

A1.1 CONSTRUCTION OF LP1 GENOMIC LIBRARY IN λ EMBL3A

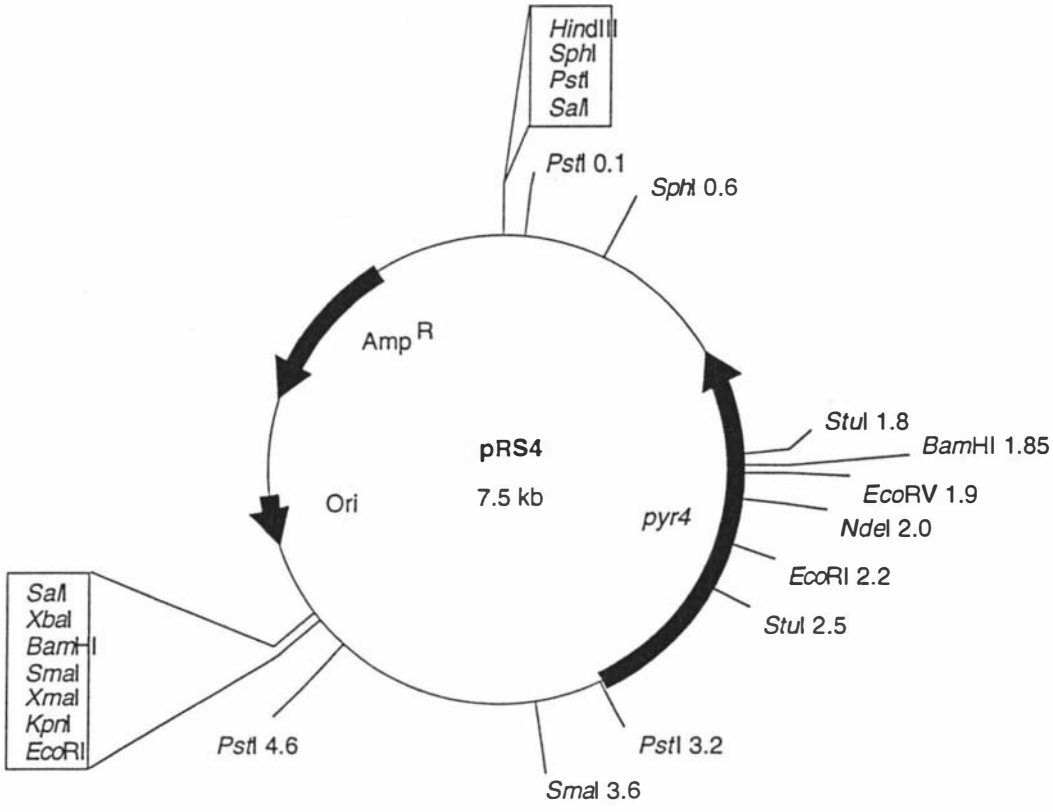
Total genomic DNA was isolated from about 1 g of freeze-dried mycelia of *Acremonium* isolate Lp1 by the method of Brownlee (1988). Genomic DNA was partially digested with *Mbo*I using standard procedures (Sambrook *et al.* 1989) to generate the maximum yield of DNA fragments in the size range 20-40 kb. The partially-digested DNA was then size-fractionated by centrifugation on a sodium chloride density gradient (Grosveld *et al.* 1982) and the fractions containing DNA fragments in the DNA size range of 20-40 kb were pooled. This fraction was dialysed in TE (10/1) and the DNA ligated to λEMBL3A *Bam*HI arms (Promega) and packaged using a Packagene system (Promega), according to the manufacturers instructions. The titre of the unamplified library was 2 x 10^8 pfu/μg of λ arms. *E. coli* host LE392 was used to titre the library. Lp1 genomic DNA was isolated by F. R. Murray. The library was constructed by C. A. Young and D. B. Scott.

A1.2 CONSTRUCTION OF LP1 GENOMIC LIBRARY IN COSMID pAN7-2

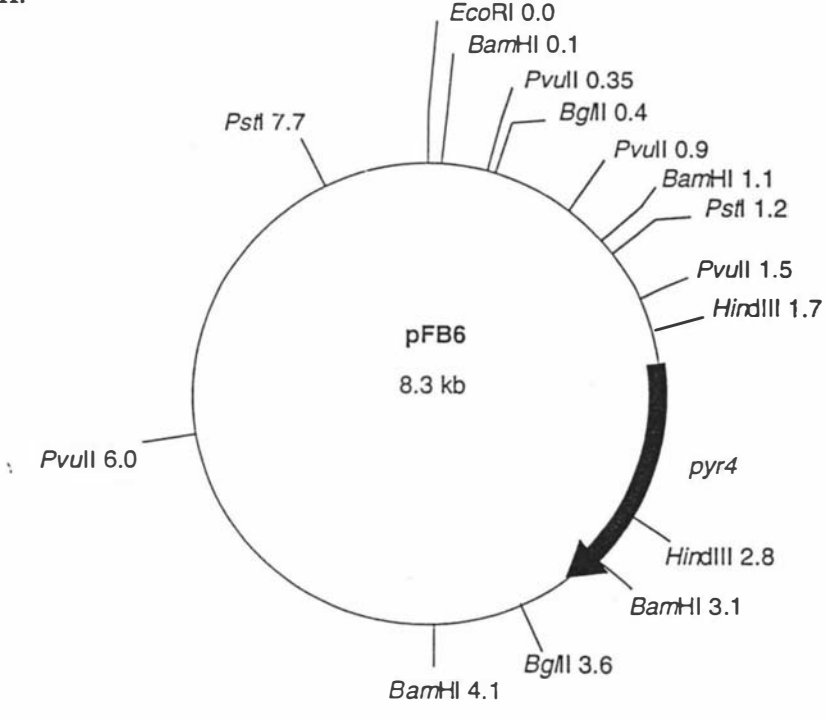
Fractionated total genomic DNA in the size range 20-40 kb, from Section A2.1, was ligated to CAP treated *Bgl*II digested pAN7-2. DNA was then packaged using a Packagene system (Promega), according to the manufacturers instructions. The library was then amplified by plating cosmids transformed into *E. coli* LE392 on LB plates supplemented with ampicillin, and making a bacterial lysate from the resulting Amp^R colonies. The titre of the library before amplification was 4 x 10^4 colonies in total.

Appendix 2.0 VECTOR MAPS

Restriction map of pRS4 showing sites for *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn* I, *Nde*I, *Pst*I, *Sal*I, *Sma* I, *Sph* I, *Stu*I, *Xba*I and *Xma* I.

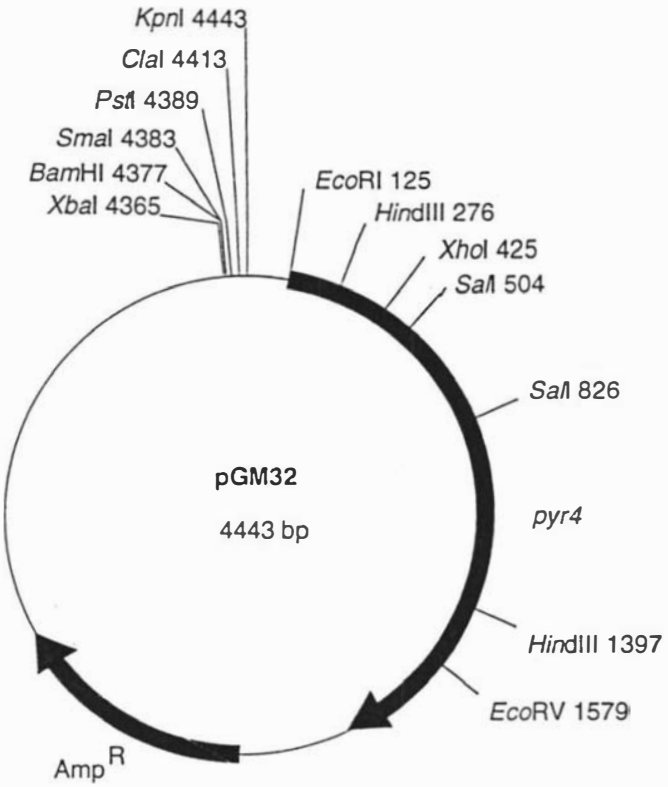


A restriction map of pFB6, showing sites for *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Pst*I and *Pvu*II.

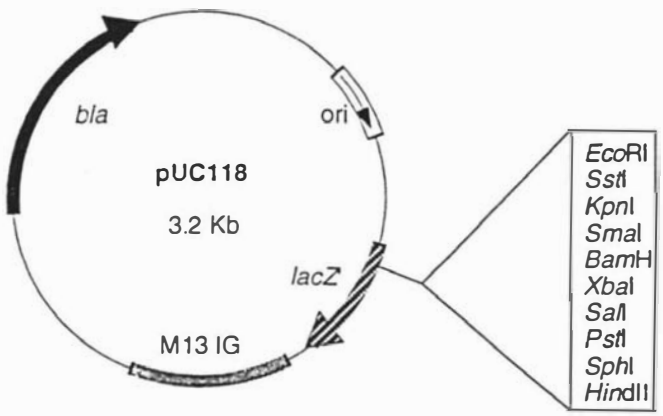


Restriction map of λ vector EMBL3A for enzymes *Bam*HI, *Bgl*II, *Eco*RI and *Sal*I.

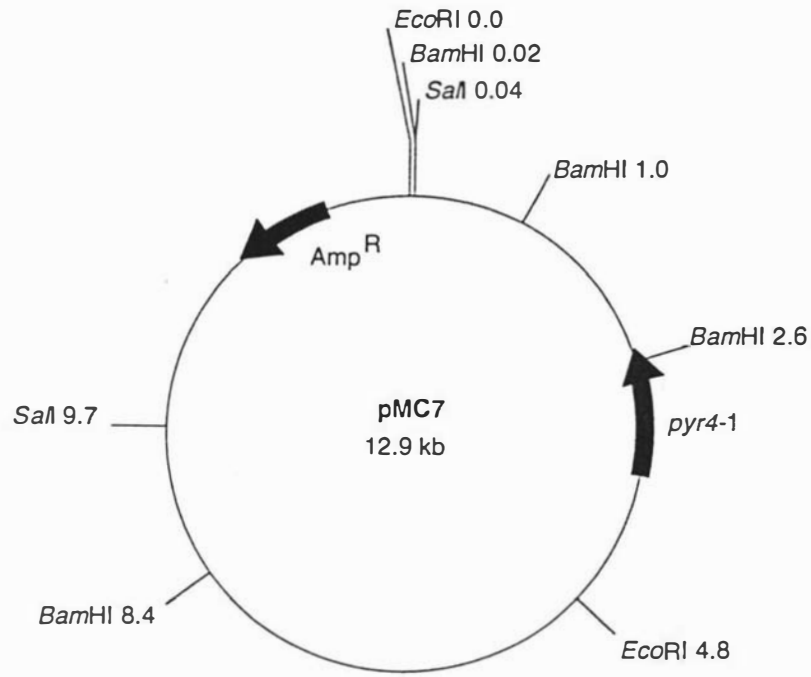
Restriction map of pGM32, showing sites for *Bam*HI, *Cla*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Xba*I and *Xho*I.



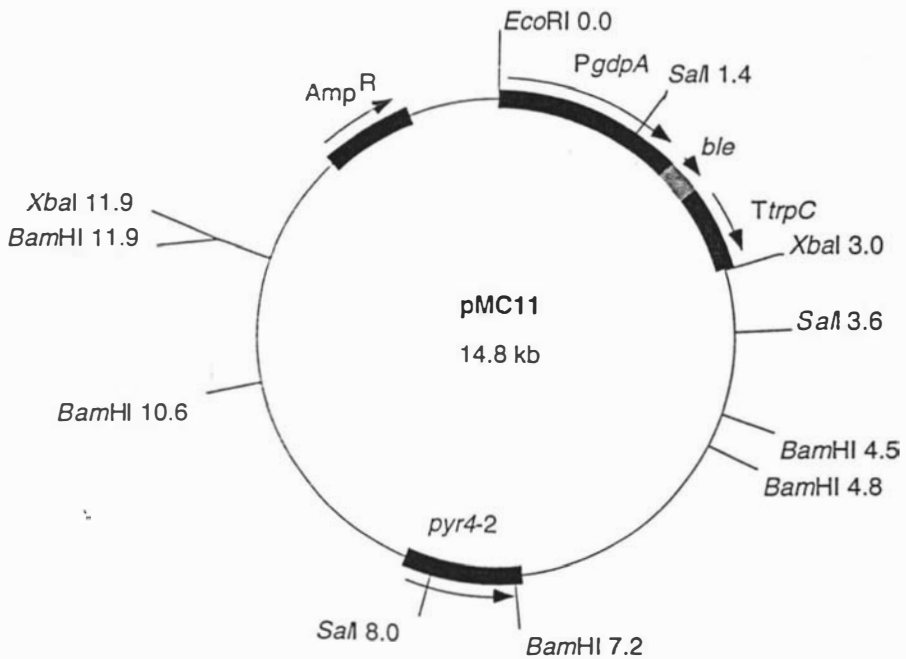
Restrictiom map of pUC118, showing restriction enzymes in the polycloning site.



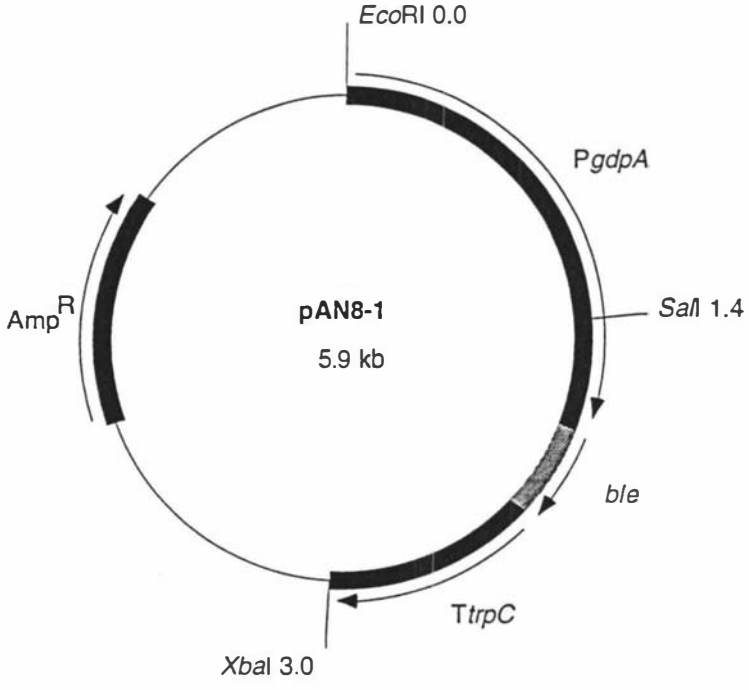
Restriction map of pMC7, showing sites for *Bam*HI, *Eco*RI and *Sal*I.



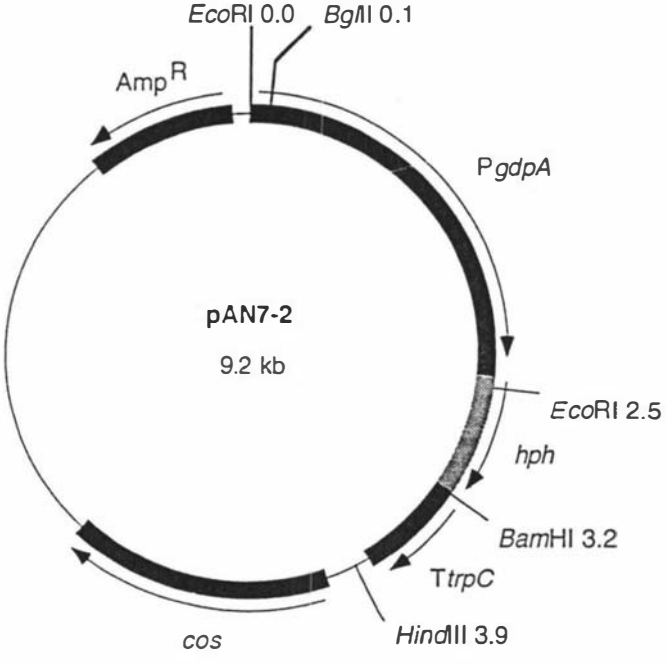
Restriction map of plasmid pMC11 showing sites for *Bam*HI, *Eco*RI, *Sal*I and *Xba*I.



A restriction map of pAN8-1 showing sites for *Eco*RI, *Sal*I and *Xba*I.



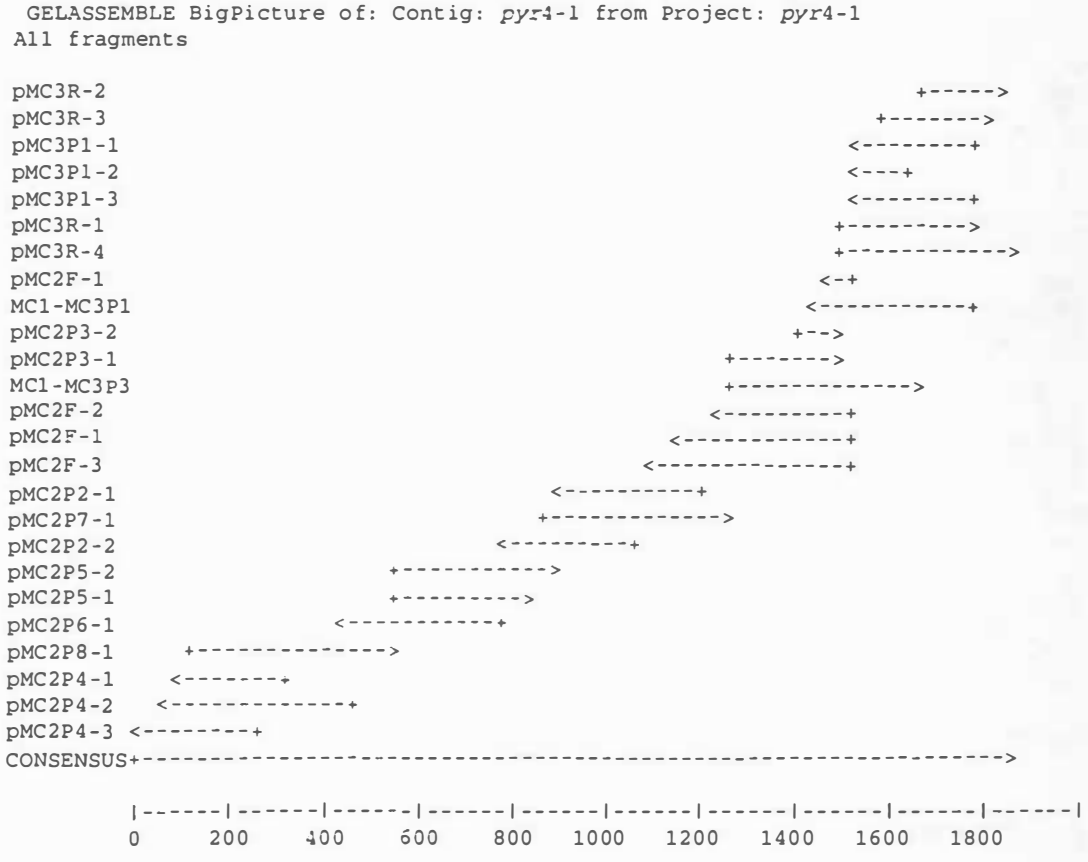
A restriction map of pAN7-2 showing sites for *Bam*HI, *Bgl*II, *Eco*RI and *Hin*dIII.

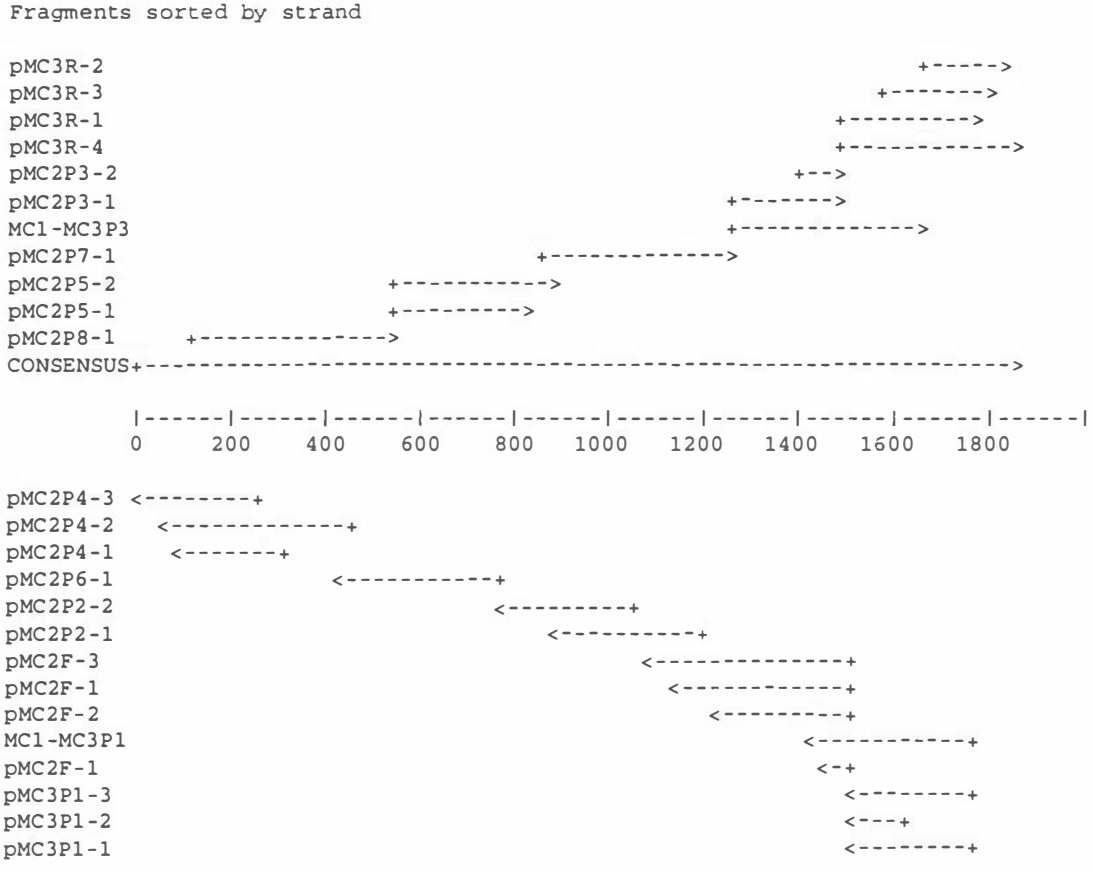


Appendix 3.0 SEQUENCE DATA

Sequence fragments are labelled by template (e.g. pMC3), followed by primer abbreviation (e.g. R for Promega reverse primer, F for Promega forward primer, and P1 for primer MC1), plasmid templates are then followed by a hyphenated number which corresponds to the occasion this template was sequenced (e.g. -2 means this sequence fragment is from the second occasion the template was sequenced). PCR products were only sequenced once. The templates for these are designated by the two primers used to amplify the template (e.g. MC1-MC3 represents a PCR product amplified with primers MC1 and MC3). Templates were amplified from the appropriate λ clone, λMC12 for *pyr*4-1 and λMC11 for *pyr*4-2. The name of the primer used for sequencing the PCR product follows the template name.

A3.1 BIGPICTURE FROM *PYR*4-1 CONTIG




Fragments sorted by strand
pMC3R-2
pMC3R-3
pMC3R-1
pMC3R-4
pMC2P3-2
pMC2P3-1
MC1-MC3P3
pMC2P7-1
pMC2P5-2
pMC2P5-1
pMC2P8-1
CONSENSUS
0
200
400
600
800
1000
1200
1400
1600
1800
pMC2P4-3
pMC2P4-2
pMC2P4-1
pMC2P6-1
pMC2P2-2
pMC2P2-1
pMC2F-3
pMC2F-1
pMC2F-2
MC1-MC3P1
pMC2F-1
pMC3P1-3
pMC3P1-2
pMC3P1-1

A3.2 PRETTYOUT FROM *PYR*4-1 CONTIG

```
 GELASSEMBLE PrettyOut of Contig: pyr4-1 from Project: pyr4-1
    From: 1  To: 1881

pMC2P4-3  < TATCACGTGGTAGGTATAGCTTTAGTAAGCAAGAAAACACAGCACCAAATAGCAACGCTT 60
CONSENSUS > TATCACGTGGTAGGTATAGCTTTAGTAAGCAAGAAAACACAGCACCAAATAGCAACGCTT 60

             .........+.........+.........+.........+.........+.........+

pMC2P4-1  <                                                  CCTAGCTATATAAGTC 16
pMC2P4-2  < GATAGTAATACATAAAGGAACTAAACATGCTTAGAAGTTAGAGGCCTAGCTATATAAGTC 60
pMC2P4-3  < GATAGTAATACATAAAGGAACTAAACATGCTTAGAAGTTAGAGGCCTAGCTATATAAGTC 120
CONSENSUS > GATAGTAATACATAAAGGAACTAAACATGCTTAGAAGTTAGAGGCCTAGCTATATAAGTC 120

             .........+.........+.........+.........+.........+.........+

pMC2P8-1  > TAAGGnAAGGgTAATTaTTACATGTAGGgATTAAgACCCCTtTncTTACTATCAGAGGTC 60
pMC2P4-1  < TAAGGAAAGGGTAATTATTACATGTAGGGATTAAGACCCCTTTTCTTACTATCAGAGGTC 76
pMC2P4-2  < TAAGGAAAGGGTAATTATTACATGTAGGGATTAAGACCCCTTTTCTTACTATCAGAGGTC 120
pMC2P4-3  < TAAGGAAAGGGTAATTATTACATGTAGGGATTAAGACCCCTTTTCTTACTATCAGAGGTC 180
CONSENSUS > TAAGGaAAGGGTAATTATTACATGTAGGGATTAAGACCCCTTTtCTTACTATCAGAGGTC 180

             .........+.........+.........+.........+.........+.........+

pMC2P8-1  > TTATTAATATGGATAACTTACTTAATCTnTCCAGGGCGATTATCCCGTAATTgGTGGACG 120
pMC2P4-1  < TTATTAATATGGATaACTTACTTAATCTGTCCAGGGCGATTATCCCgTAATTGGTGGACg 136
pMC2P4-2  < TTATTAATATGGATaACTTACTTAATCTGtCCAGGGCGATTATCCCGTAATTGGTGGACG 180
pMC2P4-3  < TTATTAATATGGATAACTTACTTAATCTGTCCAGGGCGATTATCCCGTAATTGGTGGACG 240
CONSENSUS > TTATTAATATGGATAACTTACTTAATCTgTCCAGGGCGATTATCCCGTAATTGGTGGACG 240

             .........+.........+.........+.........+.........+.........+

pMC2P8-1  > CGAAGGGGGGAAGCTGTGCCCCACTCCCCTTACGAAAATnTTTCTCCGCAGGagATAGTT 180
pMC2P4-1  < CGAAGGGGGGAAGCTGTGCCCCACTCCCCTTACGaAAATGTTTCTCCGCAGGAGATAGTT 196
pMC2P4-2  < CGAAGGGGGGAAGCTGTGCCCCACtCCCCTTACGAAAATGTTTCTCCGCAGGAGATAGTT 240
pMC2P4-3  < CGAAGGGGGGAAGCTGTGCCCCACTC                                   266
CONSENSUS > CGAAGGGGGGAAGCTGTGCCCCACTCCCCTTACGAAAATgTTTCTCCGCAGGAGATAGTT 300

             .........+.........+.........+.........+.........+.........+

pMC2P8-1  > GGGGTTtttnttttCTTcATCCTTATTAAGCTAATTgACTTTTgTTCATGAAAACATCAT 240
pMC2P4-1  < GGGGTttttTTTTTCtTCATCCTTATTAAGC                              227
pMC2P4-2  < GGGGt TTTTTTTTTCTTCATCCTTATTAAGCTAATTGACTTTTGTTCATGAAAACATCAT 300
CONSENSUS > GGGGTTTTTtTTTTCTTCATCCTTATTAAGCTAATTGACTTTTGTTCATGAAAACATCAT 360

             .........+.........+.........+.........+.........+.........+

pMC2P8-1  > CTGAGTATTGACCCTACATTgTTTCACCGTCACATTTtttTGCCGAGGAACTTGTCTACT 300
pMC2P4-2  < CTGAGTATTGACCCTACATTGTTTCACCGTCACATTTTTTTGCCGAGGAACTTGTCTACT 360
CONSENSUS > CTGAGTATTGACCCTACATTGTTTCACCGTCACATTTTTTTGCCGAGGAACTTGTCTACT 420

             .........+.........+.........+.........+.........+.........+

pMC2P6-1  <                                  TTTTACGTACCAAACGAnCCAAATATTGTG 30
pMC2P8-1  > TGCGCAAATTCAATATATCTTGACAAAGCCTTTTACGTACCAAACGATCCAAATATTGTg 360
pMC2P4-2  < TGCGCAAATTCAATATATCTTGACAAAGCCTTTTACGTAC                     400
CONSENSUS > TGCGCAAATTCAATATATCTTGACAAAGCCTTTTACGTACCAAACGAtCCAAATATTGTG 480

             .........+.........+.........+.........+.........+.........+

pMC2P6-1  < GCGAGATnGCAACCATGGCGCCGCACCAAACCCTCAAGGCGACTTACGCCTCGAGAGCTc 90
pMC2P8-1  > GCGAGATCGCAACCATGGCGCCGCACCAAACCCTCAAGGCGACTTACGCCTCGAGAGCTC 420
CONSENSUS > GCGAGATcGCAACCATGGCGCCGCACCAAACCCTCAAGGCGACTTACGCCTCGAGAGCTC 540

             .........+.........+.........+.........+.........+.........+
```

```
pMC2P5-2  >          ACACACCCgcTcAACGnCTATcTATTCAAGCTTATGGACTTGAAGGCTTCCA 52
pMC2P5-1  >       CCACACACCCGCTCAACGcCTATCTATTCAAgCTTATGGACTTgAaGGCTTCCA 54
pMC2P6-1  < AGAGTGCCAcACACCCGCTCAACGCCTATCTaTTCAAGCTTATGGACTTGAAGGCTTCCA 150
pMC2P8-1  > AGAGTGCCACACACCCGCTCAACGCCT                                  447
CONSENSUS > AGAGTGCCACACACCCGCTCAACGcCTATCTATTCAAGCTTATGGACTTGAAGGCTTCCA 600

             .........+.........+.........+.........+.........+.........+

pMC2P5-2  > ACCTCTGTtTGAGTgCTGATGTCGCGACTGCcCGGgAACTTCTTTACTTTGCCGACAAGC 112
pMC2P5-1  > ACCTCTnTtTgAnTnCTGATGTCGCGACTnCcCGGgAACTTCTTTACTTTgCCGACAAGC 114
pMC2P6-1  < ACCTCTGTTTGAGTGCTGATGTCGCGACTGCCCGGGAACTTCTTTACTTTGCCGACAAGC 210
CONSENSUS > ACCTCTgTTTGAgTgCTGATGTCGCGACTgCCCGGGAACTTCTTTACTTTGCCGACAAGC 660

             .........+.........+.........+.........+.........+.........+

pMC2P5-2  > TTGGcCCGTcTATTGTTGTTCTCAAGACACATTATGATATGGTCTCCGGCTGGGACTTCC 172
pMC2P5-1  > TTGGCCCGTnTATTgTTGTTCTCAAGACACATTATGATATGGTCTCCGGCTGGgACTTCC 174
pMC2P6-1  < TTGGCCCGTCTATTGTTGTTCTCAAGACACATTATGATATGGTCTCCGGCTGGGACTTCC 270
CONSENSUS > TTGGCCCGTcTATTGTTGTTCTCAAGACACATTATGATATGGTCTCCGGCTGGGACTTCC 720

             .........+.........+.........+.........+.........+.........+

pMC2P2-2  <                                                        TTGA 4
pMC2P5-2  > ACCCGCAAACTGGCACTGGCGCAAAGTTGGCGTCGTTAGCTCGTCGCCATGGATTCTTGn 232
pMC2P5-1  > ACCCGCAAACTGGCACTGGCGCAAAGTTGGCGTCGTTAGCtCGTcgCCATGGATTCTTGA 234
pMC2P6-1  < ACCcGCAaACTGGcACTGGcGcAAAGTTGGCGTCGTTAGCTCGTCGCCATGGATTCTTGA 330
CONSENSUS > ACCCGCAAACTGGCACTGGCGCAAAGTTGGCGTCGTTAGCTCGTCGCCATGGATTCTTGa 780

             .........+.........+.........+.........+.........+.........+

pMC2P2-2  < TATTCGAAGATCGCAAGTTCGCTGACATTGGTAACACGGTGGAGTTGCAGTACATTGGCG 64
pMC2P5-2  > TATTCGAAGATCGCAAGTtCGCTGACATTGGTAACACGGTGGAGTTGCAGTACATTGGCG 292
pMC2P5-1  > TATTCGAAGATCGCaAGTtCGCTGACATTGGTAACACGGTGGAGTTGCAGTACATTGGCG 294
pMC2P6-1  < TATTC                                                       335
CONSENSUS > TATTCGAAGATCGCAAGTTCGCTGACATTGGTAACACGGTGGAGTTGCAGTACATTGGCG 840

             .........+.........+.........+.........+.........+.........+

pMC2P7-1  >                            CACACATAGTCAATgTTAACATGGTTCCCGGAAAGG 36
pMC2P2-2  < GTTCTGCACGCATCATCGAGTGGGcACACATAGTCAATGTTAACATGGTTCCCGgAAAGG 124
pMC2P5-2  > GTTCTGCACGCATCATCGAGTGGGCACACATAGTCAATGTTAACATGGTTCCcGGAAAGG 352
pMC2P5-1  > GTTCTGCACGCATCATC                                            311
CONSENSUS > GTTCTGCACGCATCATCGAGTGGGCACACATAGTCAATGTTAACATGGTTCCCGGAAAGG 900

             .........+.........+.........+.........+.........+.........+

pMC2P2-1  <  GTCTGTCACTTCCCTGGcAAATGCTGCTACCCGGTGGTTCGAAcGGTACCCGTATGAAG 59
pMC2P7-1  > CGTCTGTcACTTCCCTGGCAAATGCTGCTACCCGGTGGTTCGAACGGTACCCGTATGAaG 96
pMC2P2-2  < CGTCTGTCACTTCCCTGGcAAATGCTGCTACCCGGTGGTTCGAACGGTACCCGTATGAAG 184
pMC2P5-2  > CGTCTG                                                       358
CONSENSUS > CGTCTGTCACTTCCCTGGCAAATGCTGCTACCCGGTGGTTCGAACGGTACCCGTATGAAG 960

             .........+.........+.........+.........+.........+.........+

pMC2P2-1  < TGAAGACGTCAGTTTCCGTGGGCACACCGACTCCGGAAGAATTTGAAgAAAATGACGCGG 119
pMC2P7-1  > TGAAGACGTCAGTtTCCGTGGGCACACCGACTCCGGAAGAATTTGAAGAAAATGACGCGG 156
pMC2P2-2  < TGAAGACGTCAGTTTCCGTGGGCACACCGACTCCGGAAGAATTTGAAGAAAATGACGCGG 244
CONSENSUS > TGAAGACGTCAGTTTCCGTGGGCACACCGACTCCGGAAGAATTTGAAGAAAATGACGCGG 1020

             .........+.........+.........+.........+.........+.........+

pMC2P2-1  < GGTCCAGCGGCCGGGACGAAGCTAACGACAAGACATCTCGGCGGCcAgACGATGGtCGTA 179
pMC2P7-1  > GGTCCAGCGGCCGGGACGAAGCTAACGACAAGACATCTCGGCGGCCAGACGATGGTCGTA 216
pMC2P2-2  < GGTCCAGCGGCCGGGACGAAGCTAACGACAAGACATCTCG                    284
CONSENSUS > GGTCCAGCGGCCGGGACGAAGCTAACGACAAGACATCTCGGCGGCCAGACGATGGTCGTA 1080

             .........+.........+.........+.........+.........+.........+
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pMC2F-3    <                    GTCACGACAGTAACACAGCAGCAATACGAACCCGTCAACTCTC 43
pMC2P2-1   < AGGGGAGCATCGTCTCCGTCACGACAGTAACACAGCAGCAATACGAACCCGTCAACTCTC 239
pMC2P7-1   > AGGGGAGCATCGTCTCCGTCACGACAGTAACACAGCAGCAATACGAACCCGTCAACTCTC 276
CONSENSUS > AGGGGAGCATCGTCTCCGTCACGACAGTAACACAGCAGCAATACGAACCCGTCAACTCTC 1140

             .........+.........+.........+.........+.........+.........+

pMC2F-1    <                  CATGACTgGCGGGGATGAGGTTCTATTTGCTGGCATCgAGGAGG 44
pMC2F-3    < CTCGACTGACCAAAAGCATGACTGGCGGGGATGAGGTTCTATTTGCTGGCATCGAGGAGG 103
pMC2P2-1   < CTCGACTGACcAAAAGCATnACTGGCGGGGATGAGGTTCtATTnGCTGGCATCGAGGAGG 299
pMC2P7-1   > CTCGACTGACCAAAAGCATGACTGGCGGGGATGAGGTTCTATTTGCTGGCATCGAGGAGG 336
CONSENSUS > CTCGACTGACCAAAAGCATgACTGGCGGGGATGAGGTTCTATTtGCTGGCATCGAGGAGG 1200

             .........+.........+.........+.........+.........+.........+

pMC2F-2    <                                    ACAGATGTCCTCgGCGGGTAATTTTATGA 29
pMC2F-1    < CACCTATGGcGCGAGGTCTACTCATTCTGGcACAGATGTCCTCGGCGGGTAATTTTATGA 104
pMC2F-3    < CACCTATGGCGCGAGGTCTACTCATTCTGGCACAGATGTCCTCGGCGGGTAATTTTATGA 163
pMC2P2-1   < CACCTATGGCGCnAGGTCnAC                                        320
pMC2P7-1   > CACCTATGGCGCGAGnTCTACTCATTCTGGCACAGATGTCCTCGGCGGGTAATTTTATGA 396
CONSENSUS > CACCTATGGCGCgAGgTCtACTCATTCTGGCACAGATGTCCTCGGCGGGTAATTTTATGA 1260

             .........+.........+.........+.........+.........+.........+

pMC2P3-1   >      GGnGTATACACAaGCnTGCgTGGnGGCTGCCAGAgAACACAAAGATTTtntnATGG 56
MC1-MC3P3  >      GGAGTATACACAAGCTTGCgTGGaGGCTGCCAGAGAaCACAAAGATTTTGTCATGG 56
pMC2F-2    < ATAAGGAGTATAcACAaGCTTgCGTGGAGGCTGCCAGAGAAcACAAAGATTTTGTCATGG 89
pMC2F-1    < ATAAGGaGTATACACAAGCTTgCGTGGAGGCTGCCAGaGaACACAAAGATTTTGTCATGG 164
pMC2F-3    < ATAAGGAGTATACACAAGCTTGCGTGGAGGCTGCCAGAGAACACAAAGATTTTGTCATGG 223
pMC2P7-1   > ATAAGGAGTATACACAAGCTT                                        417
CONSENSUS > ATAAGGaGTATACACAAGCtTGCGTGGaGGCTGCCAGAGAACACAAAGATTTTgTcATGG 1320

             .........+.........+.........+.........+.........+.........+

pMC2P3-1   > GATTtGTATCACAAGAgACGCTGAATACGGAACCCGATGACAAAtttATTCACATGACGC 116
MC1-MC3P3  > GATTTGTATCACAAGAGACGCTGAATACGGAACCCGATGACAAATTTATTCACATGACGC 116
pMC2F-2    < GATTTGTATCACAAGAGACGCTGAATACGGAACCCGATGACAAATTTATTCACATGACGC 149
pMC2F-1    < gATTTGTATCACAAGAGACGCTGAATACGGAACCCGATGACAAATTTATTCACATGACGC 224
pMC2F-3    < GATTTGTATCACAAGAGACGCTGAATACGGAACCCGATGACAAATTTATTCACATGACGC 283
CONSENSUS > GATTTGTATCACAAGAGACGCTGAATACGGAACCCGATGACAAATTTATTCACATGACGC 1380

             .........+.........+.........+.........+.........+.........+

pMC2P3-2   >                                        GCACGGTCCAGGGCGACGGAA 21
pMC2P3-1   > CAGGCTGCCAGCTGCCACCCGAAGACGAGGATCAGAACGGCACGGTCCAGGGCGACGGAA 176
MC1-MC3P3  > CAGGCTGCCAGCTGCCACCCGAAGACGAGGATCAGAACGGCACGGTCCAGGGCGACGGAA 176
pMC2F-2    < CAGGCTGCCAGCTGCCACCCGAAgACGAGGaTCAgAACGGcACGGTCCAGGGCGACGgnA 209
pMC2F-1    < CAGGCTGCCAGCTGCCACCCGAAgACGAGGATCAGAACGGcACGGTCCAGGGCGACGgnA 284
pMC2F-3    < CAGGCTGCCAGCTGCCACCCGAAGACGAGGATCAGAACGGCACGGTCCAGGGCGACGGAA 343
CONSENSUS > CAGGCTGCCAGCTGCCACCCGAAGACGAGGATCAGAACGGCACGGTCCAGGGCGACGGaA 1440

             .........+.........+.........+.........+.........+.........+

pMC2F-1    <                                    ATCGGCGTAGCTGGAGCGGACATCGTCA 28
MC1-MC3P1  <        AGCAGTATAATACACCACAAAAGATTATCGGCGTAGCTGGAGCGGACATCGTCA 54
pMC2P3-2   > AAGGCCAGCAGTATAATACACCACAAAAGATTATCGGCGTAgcTGGAGCGGACATCGTCA 81
pMC2P3-1   > AAGGCCAGCAGTATAATACACCACAAAAGATTATCGGCGTAgcTGGAGCgGACATCGTCA 236
MC1-MC3P3  > AAGGCCAGCAGTATAATACACCACAAAAGATTATCGGCGTAgcTGGAGCGGACATCGTCA 236
pMC2F-2    < AAGGCCAGCAGTATAATACACcACAAAAgATTATCGGCGTAGCTGGagCGGACATCGTCA 269
pMC2F-1    < AAGgCCAGCAGTATAATACACCACAAAAGATTATCGGCGTAGCTGGaGCGgACATCGTCA 344
pMC2F-3    < AAGGCCAGCAGTATAATACACcACAAAAnATTATCGGCGTAGCTGGAGCGGACATCGTcA 403
CONSENSUS > AAGGCCAGCAGTATAATACACCACAAAAgATTATCGGCGTAGCTGGAGCGGACATCGTCA 1500

             .........+.........+.........+.........+.........+.........+
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pMC3P1-1  <                      TAAaGCAGGGGACCCCGAGGGCGAAACGGAGCGATATCGCT 41
pMC3P1-2  <                   CCTTAAAGCAGGGGACCCCGAGGGCGAAACGGAGCGATATCGCT 44
pMC3P1-3  <                   CCTTAAAGCAGGGGACCCCGAGGGCGAAACGGAgCGATATCGCT 44
pMC3R-1   >              GGATCCTTAAAGCAGGGGACCCCGAGGGCGAAACGGAGCGATATCgcT 48
pMC3R-4   >              GGATCCTTAaaGCaGGGGACCCCGAgGgCGAAAcggAGCgATATCGCt 48
pMC2F-1   < TTGTCGGCCGTGGGATCC                                             46
MC1-MC3P1 < TTGTCGGCCGTGGGATCCTTAAAGCAGGGGACCCCGAGGGCGAAACGGAGCGATATCgCT 114
pMC2P3-2  > TTGTCGGCCGT                                                     92
pMC2P3-1  > TTGTCGGCCGT                                                    247
MC1-MC3P3 > TTGTCGGCCGTGGGATCCTTAAAGCAGGGGACCCCGAGGGCGAAACGGAGCGATATCGCT 296
pMC2F-2   < TTGTCGGCCGTGGGATCC                                             287
pMC2F-1   < TTGTCGgCCGTGGGATCC                                             362
pMC2F-3   < TTGTCGGCCGTGGGATCC                                             421
CONSENSUS > TTGTCGGCCGTGGGATCCTTAAAGCAGGGGACCCCGAGGGCGAAACGGAGCGATATCGCT 1560

             .........+.........+.........+.........+.........+.........+

pMC3R-3   >              GGgTTACACTGAGAGGgTTCGCTAGCaACTTGAGCTAGGTgGCTGGC 47
pMC3P1-1  < CTGCGGCTTGGAAGGGTTACACTGAGAGGGTTCGCTAGCAACTTGAGCTAGGTGGCTGGC 101
pMC3P1-2  < CTGCGGCTTGGAAGGGTTACACTGAGAGGGTTCGCTAGCAACTTGAGCTAGGTGGCTGGC 104
pMC3P1-3  < CTGCgGCTTGGAAGGGTTACACTGAGAGGGTTCGCTAGCaACTTGAGCTAGGTGGCTGGC 104
pMC3R-1   > cTGcgGCTTGGAAGGGTTACACTGAGAGGGTTCGCTAGCAACTTGAGCTAGGTGGCTGGc 108
pMC3R-4   > CTGCgGCTTGGaAGGgTTACACTGAGAGGgTTCGCTAGCAACTTGAgCTAGGTGGCTGGC 108
MC1-MC3P1 < CTGCGGCTTGGAAGGGTTACACTGAGAGGGTTCGCTAGCAACTTGAGCTAGGTGGCTGGC 174
MC1-MC3P3 > CTGCGGCTTGGAAGGGTTACACTGAGAGGGTTCGCTAGCAACTTGAGCTAGGTGGCTGGC 356
CONSENSUS > CTGCGGCTTGGAAGGGTTACACTGAGAGGGTTCGCTAGCAACTTGAGCTAGGTGGCTGGC 1620

             .........+.........+.........+.........+.........+.........+

pMC3R-2   >                                                  AAGAAGCATAAAC 13
pMC3R-3   > CATTGGTTCTgAaAATtTTgCtTGAaACTaACCTGGCATTTTgaaGGaaGaaGCATAAAC 107
pMC3P1-1  < CATTGGTTCtGaaaaTTTTGCTTGAAACTAACCTGGCATTTTGAAGGnAGaagcATAaAC 161
pMC3P1-2  < CATTGGTTCTgAAAA                                                119
pMC3P1-3  < CATTGGTTCTgAAAATTTTGCTTGAAACTAACCTGGCATTTTGAAGGnAGAAGcATAaAC 164
pMC3R-1   > CATTGGTTCTGAAAATTTTnCTTGAAACTAACCTGGCATTTTGaagGAAGAAGCATAAAC 168
pMC3R-4   > CATTgGTTCTgAaAATtTTnCTTGAaACTAACCTGGCATTTTGAAGGAAGAAGCATAAAC 168
MC1-MC3P1 < CATTGGTTCTGAAAATTTTGCTTGAAACTAACCTGGCATTTTGAAGGAAGAAGCATAAAC 234
MC1-MC3P3 > CATTGGTTCTGAAAATTTTGCTTGAAACTAACCTGGCATTTTGAAGGAAGAAGCA      411
CONSENSUS > CATTGGTTCTGAAAATTTTgCTTGAAACTAACCTGGCATTTTGAAGGaAGAAGCATAAAC 1680

             .........+.........+.........+.........+.........+.........+

pMC3R-2   > ATGGGGAAAATAGGAGGTACGCATCATTCTCTGTTTCGGCATCATGCGTTACGcTTAGCT 73
pMC3R-3   > ATGGgGAAAATAGGAGGTACGCaTCAtTCTCTgTtTCgGCATCATGCgTTACGCTTAGCT 167
pMC3P1-1  < ATGGGnnAAATAGnAGGTACgCATCATTCTcTGTTTCGGCATCATGCGTTAcGCTTAGCT 221
pMC3P1-3  < ATGGGgaAaATAGGAGGTAcGCATCATTcTcTGTTTCGGCATCATGCGTTAcGCTTAGCT 224
pMC3R-1   > ATGGGGAAAATAGGAGGTACgCATCATTCTCTGTTTCGGCATCATGCGTTAC.nTTAGCT 228
pMC3R-4   > ATGGGGAAAATAGGAGGTACGCaTCAtTCTCTGTtTCGGCATCATGCgTTACGCTTAGCT 228
MC1-MC3P1 < ATGGGGAAAATAGGAGGTACGCATCATTCTCTGTTTCGGCATCATGCGTTACGCTTAGCT 294
CONSENSUS > ATGGGgaAAATAGgAGGTACGCATCATTCTCTGTTTCGGCATCATGCGTTACgcTTAGCT 1740

             .........+.........+.........+.........+.........+.........+

pMC3R-2   > AATTATGGAATACAAGCATCCTTCCCCCCGnnCCTTTTTGACAACAGCCAACTTCCTAAT 133
pMC3R-3   > AATTATgGaATACAAGCaTCCTTCCCCCCGatCCTttttgACAACAGCCAACTTCCTAAT 227
pMC3P1-1  < AATTATGGAATaCAAGCATCCTTCCCcCCGAnCCtnTTTgACaACAgCCnAC          273
pMC3P1-3  < AATTATGGAAnACAAGCATCCTTCCCcCCGAnccnnttTgACnACAGC              272
pMC3R-1   > AATTATGGAATACAAGCATCCTTCCCCCCGnTCCTTTTTGACAAC                 273
pMC3R-4   > AATTATGGAATACAAGCaTCCTTCCCCCCGatCCTTTTTGACAACAGCCAACTTCCTAAT 288
MC1-MC3P1 < AATTATGGAATACAAGCATCCTTCCCCCCGAnCC                            328
CONSENSUS > AATTATGGAAtACAAGCATCCTTCCCCCCGatCCttTTTGACaACAGCCaACTTCCTAAT 1800

             .........+.........+.........+.........+.........+.........+

pMC3R-2   > CTGCAATGGCATTAGGCTTTGAAGAATATGGATCGCcGTCTGGCTGC              180
pMC3R-3   > CTGCaATGGCATTAGGCTT                                        246
pMC3R-4   > CTGCaATGGCATTAGGCTTTGAAGAATATGGaTCGCCGTCTGGCTGCATTCTTTCTTACC 348
CONSENSUS > CTGCAATGGCATTAGGCTTTGAAGAATATGGATCGCCGTCTGGCTGCATTCTTTCTTACC 1860

             .........+.........+.........+.........+.........+.........+
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pMC3R-4    > CTCTTGGaTCTGGAGCACAAA                                    369
CONSENSUS  > CTCTTGGATCTGGAGCACAAA                                    1881

             .........+.........+.........+.........+.........+.........+
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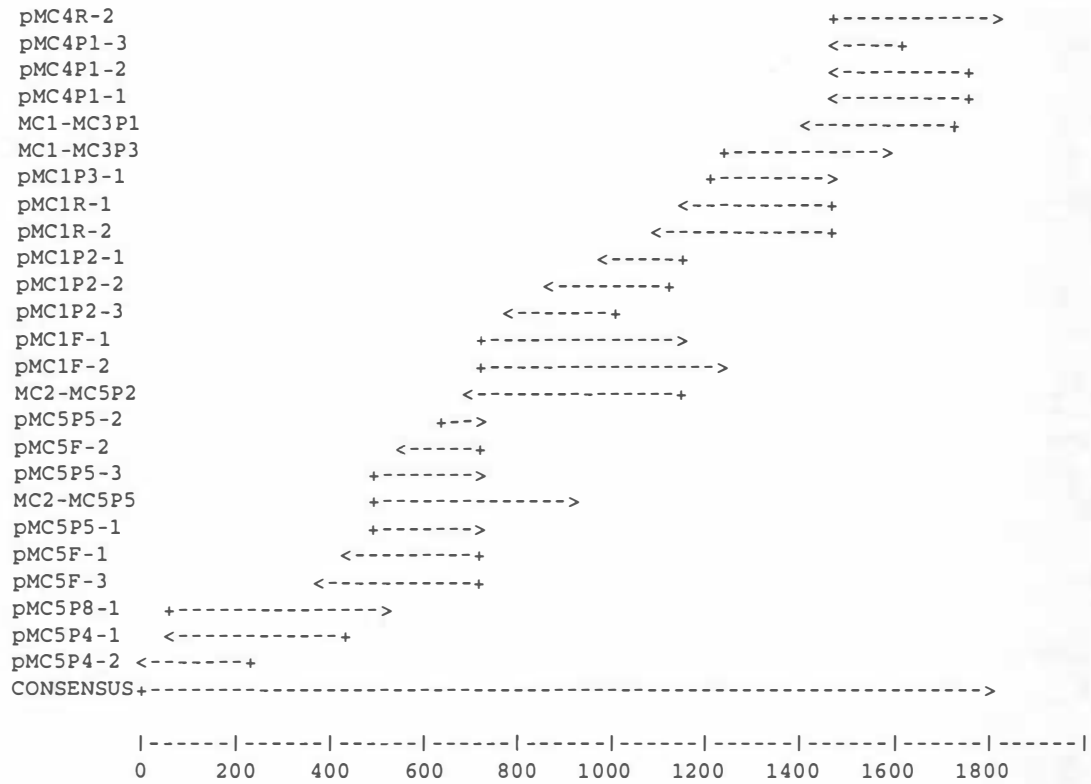
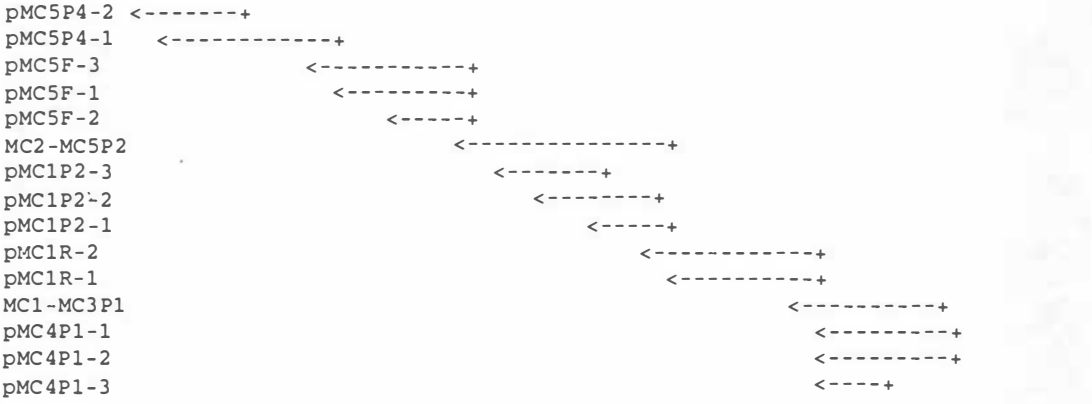
A3.3 BIGPICTURE FROM *PYR*4-2 CONTIG

GELASSEMBLE BigPicture of: Contig: *pyr*4-2 from Project: *pyr*4-2
All fragments



Fragments sorted by strand



A3.4 PRETTYOUT FROM *PYR*4-2 CONTIG

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 GELASSEMBLE PrettyOut of Contig: pyr4-2 from Project: pyr4-2
    From: 1  To: 1800

pMC5P4-2  < CATAGCTCCAAATAGCAACGCTTAAGAGTAATACAGGAACTAAACATGCTTAGAAGCCTA 60
CONSENSUS > CATAGCTCCAAATAGCAACGCTTAAGAGTAATACAGGAACTAAACATGCTTAGAAGCCTA 60

             .........+.........+.........+.........+.........+.........+

pMC5P8-1  >                          AATTATTAGGGATTATnTtATCAAGGgTTAAACCCC 36
pMC5P4-1  <               AAGGAAAGTGGAATTATTAGGGATTATGtTATCAAGGGtTAAACCCC 47
pMC5P4-2  < GCTATTTAAGTCTAAGGAAAGTGGAATTATTAGGGATTATGTTATCAAGGGTTAAACCCC 120
CONSENSUS > GCTATTTAAGTCTAAGGAAAGTGGAATTATTAGGGATTATgTTATCAAGGGTTAAACCCC 120

             .........+.........+.........+.........+.........+.........+

pMC5P8-1  > CTtnnnACTATTAGAGGGCTTATTAATACATATGGaTAACTTACTCaaTCTgTCTATGTa 96
pMC5P4-1  < CtTTTTACTATTAGAGGGCTTATTAATACATATGGATAACTTACTCAATCTGTCTATGTA 107
pMC5P4-2  < CTTTTTACTATTAGAGGGCTTATTAATACATATGGATAACTTACTCAATCTGTCTATGTA 180
CONSENSUS > CTTtttACTATTAGAGGGCTTATTAATACATATGGATAACTTACTCAATCTGTCTATGTA 180

             .........+.........+.........+.........+.........+.........+

pMC5P8-1  > AGGCGACTATCCTGTaTGTaGTGGTAAACGCGAAGGGGGGGAGGTGGACCCCACTCCCCT 156
pMC5P4-1  < AGGCGACTATCCTGTATGTAGTGGTAAACgCgAAGGGGGGGAGGTGGACCCCACTCCCCT 167
pMC5P4-2  < AGGCGACTATCCTGTATGTAGTGGTAAACGCGAAGGGGGGGAGGTGGACCC          231
CONSENSUS > AGGCGACTATCCTGTATGTAGTGGTAAACGCGAAGGGGGGGAGGTGGACCCCACTCCCCT 240

             .........+.........+.........+.........+.........+.........+

pMC5P8-1  > TATGAaAATnTtTCTCCGCAGGTtTttttttttcttCTCTACCTCATAATAAGAAGCTAA 216
pMC5P4-1  < TATGAAAATGTTTCTCCGCAGGTTTTTTTTTTCTTCTCTACCTCATAATAAGAAGCTAA 227
CONSENSUS > TATGAAAATgTTTCTCCGCAGGTTTTTTTTTTCTTCTCTACCTCATAATAAGAAGCTAA 300

             .........+.........+.........+.........+.........+.........+

pMC5P8-1  > TTGACTTTTgTnTATGAAACCATCATCTGGGTATTGACCCTGCATTGTtTCATCGTCAAA 276
pMC5P4-1  < TTGACTTTTGTTTATGAAACCATCATCTGGGTATTGACCCTGCATTGTTTCATCGTCAAA 287
CONSENSUS > TTGACTTTTGTtTATGAAACCATCATCTGGGTATTGACCCTGCATTGTTTCATCGTCAAA 360

             .........+.........+.........+.........+.........+.........+

pMC5F-3   <                                        CAGTATATATTgACAAAGCCttTT 24
pMC5P8-1  > TTTTATTGTCAAGAAACTTATTGTCTTGCACAAATTCAGTATATATTGACAAAGCCTTTT 336
pMC5P4-1  < TTTTATTGTCAAGAAACTTATTGTCTTGCACAAATTCAGTATATATTGACAAAGCCTTTT 347
CONSENSUS > TTTTATTGTCAAGAAACTTATTGTCTTGCACAAATTCAGTATATATTGACAAAGCCTTTT 420

             .........+.........+.........+.........+.........+.........+

pMC5F-1   <                CAAATATTGTGGCGAGAnnGCAGCCATGGCACCGCACCAAACCCT 45
pMC5F-3   < ACCCACCAAaAGAntcAAATATTGTGGCGAGAnnGCAGCCATGGCACCGCACCAAACCCT 84
pMC5P8-1  > ACCCACCAAAAGATTCAAATATTGTGGCGAGATCGCAGCCATGGCACCGCACCAAACCCT 396
pMC5P4-1  < ACCCACCAAAAGAT                                              361
CONSENSUS > ACCCACCAAAAGAtTCAAATATTGTGGCGAGAtcGCAGCCATGGCACCGCACCAAACCCT 480

             .........+.........+.........+.........+.........+.........+

pMC5P5-3  >                                GTGCCACACACCCGCTnAACGnCTATCTATT 31
MC2-MC5P5 >                                GTGCCACACACCCgnTCAACGCCTATnTATT 31
pMC5P5-1  >                       GAGcnCAGAGTGCCACAnACCCGCTCAACGCCTATnTATT 40
pMC5F-1   < CAAGGCgACTTACGCCTCGAGAGCTCAGAGTGCCAcACACCCGCTCAACGCCTATCTATT 105
pMC5F-3   < CAAGGCgACTTACGCCTCGAGAGCTCAGAGTGCCAcACACCCGCTCAACGCCTATCTAtT 144
pMC5P8-1  > CAAGGCGACTTACGCCTCGAGAGCTCAGAGTGCCACACACCCG                  439
CONSENSUS > CAAGGCGACTTACGCCTCGAGAGCtCAGAGTGCCACAcACCCGcTcAACGcCTATcTATT 540

             .........+.........+.........+.........+.........+.........+
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pMC5F-2    <                                CCTCTGTTTGAGTGCTGATGTCGCGACTGCCCG 33
pMC5P5-3   > CAAGCTTATGGACTTGAAGGCGTCCAACCTCTGTtTGAGTgCTGATGTCGCGACTGCCCG 91
MC2-MC5P5  > CAAGCtTATGGACTTGAAGGCGTCCAACCTCTGTtTGaGTGCTGATGTCGCGACTGCCCG 91
pMC5P5-1   > CAAGCnTATGGACTTGAaGGCGTCCAACCTcTGTnTGAGTgCTGATGTCGCGACTGCnCG 100
pMC5F-1    < CAAGCTTATGGACTTGAAGGCGTCCAACCTCTGTTTGAGTGCTGATGTCGCGACTGCCCG 165
pMC5F-3    < CAAGCTTATGGACttGAAGGCGTCCAACCTCTGTTTGAGTGCTGATGTcGCGACTGCCCG 204
CONSENSUS  > CAAGCtTATGGACTTGAAGGCGTCCAACCTCTGTtTGAGTGCTGATGTCGCGACTGCcCG 600

             .........+.........+.........+.........+.........+.........+

pMC5P5-2   >                                                    AaGAcGCACTA 11
pMC5F-2    < GGAACTTCTTTACTTTGCCGACAAGCTTGGCCCGTCTATCGTTGTTCTCAAGACGCACTA 93
pMC5P5-3   > GGAACTTCTTTACTTTGCCGACAAGCTTGGCCCGTcTATCGTTGTTCTCAAGACGCACTA 151
MC2-MC5P5  > GGAACTTCTTTACTTTGCCGACAAGCTTGGCCCGTCTATCGTTGTTCTCAAGACGCACTA 151
pMC5P5-1   > GGAACTTCTTTACTTTGCCGACAAGCTTGGCCCGTnTATCGTTGTnCTCAAGACGCACTA 160
pMC5F-1    < GGAACTTCTTTACTTTGCCGACAAGCTTGGCCCGTCTATCGTTGTTCTCAAGACGCACTA 225
pMC5F-3    < GGAACTTCTTTACTTTgCCgACAAGCTTGGCCCGTCTATCGTTGTTCTCAAGACGCACTA 264
CONSENSUS  > GGAACTTCTTTACTTTGCCGACAAGCTTGGCCCGTcTATCGTTGTtCTCAAGACGCACTA 660

             .........+.........+.........+.........+.........+.........+

MC2-MC5P2  <                                      AACTGGCACTGGCGCAAAGTTGGCGTC 27
pMC5P5-2   > TGATATGGTCTCCGGCTGGGACTTCCACCCGCAAACTGGCACTGGCGCAAAGTTGGCGTC 71
pMC5F-2    < TGATATGGTCTCCGGCTGGGACTTCCACCCGCAAACTGGCACTGGcGcAAAGTTGGCGTC 153
pMC5P5-3   > TGATATGGTCTCCGGCTGGGACTTCCACCCGCAAACTGGCACTGGCGCAAAGTTGGCGTC 211
MC2-MC5P5  > TGATATGGTCTCCGGCTGGGACTTCCACCCGCAAACTGGCACTGGCGCAAAGTTGGCGTC 211
pMC5P5-1   > TGATATGGTCTCCGGCTGGGACTtcCACCCGCAAACTGGCACTGGCGCAAAGTTGGCGTC 220
pMC5F-1    < TGATATGGTCTCCGGCTGGgACTTCCACCcGCAAACTGGcACTGGcGCAAAGTTGGCGTC 285
pMC5F-3    < TGATATGGTCtCCGGCTGGgACTtCcACCcGCAaACTGGcACTGGcGcAAAGTTGGCGtC 324
CONSENSUS  > TGATATGGTCTCCGGCTGGGACTTCCACCCGCAAACTGGCACTGGCGCAAAGTTGGCGTC 720

             .........+.........+.........+.........+.........+.........+

pMC1P2-3   <                                                      CATTGGTAA 9
pMC1F-1    >          GTCGACATGGATTCTTGATTTTCGAAGATCGCAAGTtCGCTGACATTGGTAA 52
pMC1F-2    >          GTCGaCATGGaTTCTTGATTTTCGAAGATCGCAAGTtCGCTGACATTGGTAA 52
MC2-MC5P2  < GTTAGCTCGTCGACATGGATTCTTGATTTTCGAAGATCGCAAGTTCGCTGACATTGGTAA 87
pMC5P5-2   > GTTAGCTCGTCGAC                                              85
pMC5F-2    < GTTAGCTCGTCGAC                                              167
pMC5P5-3   > GTTAGCTC                                                    219
MC2-MC5P5  > GTTAGCTCGTCGACATGGATTCTTGATTTTCGAAGATCGCAAGTTCGCTGACATTGGTAA 271
pMC5P5-1   > GTTAGCtCGTCGAC                                              234
pMC5F-1    < GTTAGCTCGTCGAC                                              299
pMC5F-3    < GTTAGCtCGTCGAC                                              338
CONSENSUS  > GTTAGCTCGTCGACATGGATTCTTGATTTTCGAAGATCGCAAGTTCGCTGACATTGGTAA 780

             .........+.........+.........+.........+.........+.........+

pMC1P2-3   < CACGGTGGAGTTgcAGTACATTgGCGGTTCTGCGCGCATCATCGAGTGGGcACACATAGT 69
pMC1F-1    > CACGGTGGAGTTGCAGTACATTGGCGGTTCTgCgCGCATCATCGAGTGGGCACACATAGT 112
pMC1F-2    > CACGGTGGAGTTGCAGTACATTGGCGGTTCTGCGCGCATCATCGAGTGGGCACACATAGT 112
MC2-MC5P2  < CACGGtGGAGTTGCAGTACATTGGCGGTTCTGCGCGCATCATCGAGTGGGCACACATAGT 147
MC2-MC5P5  > CACGGTGGAGTTGCAGTACATTGGCGGTTCTGCGCGCATCATCGAGTGGGCACACATAGT 331
CONSENSUS  > CACGGTGGAGTTGCAGTACATTGGCGGTTCTGCGCGCATCATCGAGTGGGCACACATAGT 840

             .........+.........+.........+.........+.........+.........+

pMC1P2-2   <                         AAAGGCGTCTGTCACTTCCCTGGCAAATGCTGCTACCCG 39
pMC1P2-3   < CAATGTTAACATGGTTCCCGnAAAGGCGTCTGTCACTTCCCTGGnAAATGCTGCTACCCG 129
pMC1F-1    > CAATGTTAACATGGTTCCCGGAAAGGCGTCTGTCACTTCCCTGGCAAATGCTGCTACCCG 172
pMC1F-2    > CAATGTTAACATGGTTCCCGGAAAGGCGTCTGTCACTTCCCTGGCAAATGCTGCTACCCG 172
MC2-MC5P2  < CAATgTTAACATGGTTCCCGgAAAGGCGTCTGTCACTTCCCTGGCAAATGCTGCTACCCG 207
MC2-MC5P5  > CAATGTTAACATGGTTCCCGGAAAGGCGTCTGTCACTTCCCTGGCAAATGCTGCTACCCG 391
CONSENSUS  > CAATGTTAACATGGTTCCCGgAAAGGCGTCTGTCACTTCCCTGGcAAATGCTGCTACCCG 900

             .........+.........+.........+.........+.........+.........+
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pMC1P2-2  < GTGGTTCGAACGGTACCCGTATGAAGTGAAGACGTCAGTTTCCGTGGGCACACCGACTGC 99
pMC1P2-3  < GTGGTTCGAACGGTACCCGTATGAAGTGAAGACGTCAGTTTCCGTGGGcACACCGACTGC 189
pMC1F-1   > GTGGTTCGAACGGTACCCGTATGAAGTGAAGACGTCAGTTTCCGTGGGCACACCGACTGC 232
pMC1F-2   > GTGGTTCGAACGGTACCCGTATGAAGTGAAGACGTCAGTTTCCGTGGGCACACCGACTGC 232
MC2-MC5P2 < GTGGTTCGAACGGTACCCGTATGAAGTGAAGACGTCAGTTTCCGTGGGCACACCGACTGC 267
MC2-MC5P5 > GTGGTTCGAACGGTACCCGTATGAAGTGAAGACGTCA                         428
CONSENSUS > GTGGTTCGAACGGTACCCGTATGAAGTGAAGACGTCAGTTTCCGTGGGCACACCGACTGC 960

          .........+.........+.........+.........+.........+.........+

pMC1P2-1  <                       CgCGGGGTCCAgCGGCCGGGnCGAAGCTAATGGCAATAC 39
pMC1P2-2  < GGAAGACTTTGAAGAAAATGACGCGGGGTCCAGCGGCCGGGACGAAGCTAATGGCAATAC 159
pMC1P2-3  < GGAAgACTTTGaAgaAAATgACGCGGGGTCCAGCGGCCGGnaCGAAGCTAAT         241
pMC1F-1   > gGAAGACTTTGAAGAAAATGACGCGGGGTCCAGCGGCCGGGACGAAGCTAATGGCAATAC 292
pMC1F-2   > gGAAGACTTTGAAGAAAATGACGCGGGGTCCAGCGGCCGGGACGAAGCTAATGGCAATAC 292
MC2-MC5P2 < GGAAGACTTTGAAgAAAATGACGCGGGGTCCAGCGGCCGGGACGAAGCTAATGGCAATAC 327
CONSENSUS > GGAAGACTTTGAAGAAAATGACGCGGGGTCCAGCGGCCGGgaCGAAGCTAATGGCAATAC 1020

          .........+.........+.........+.........+.........+.........+

pMC1P2-1  < ATCTCGGCGnCCAGACGATGnTCGTAAGGGGAGCATCGTCTCCGTCACgACAGTAAcACA 99
pMC1P2-2  < ATCTCGGCGGCCAGACGATGGtCGTAAGGGGAGCATCGTCTCCGTCACGACAGTAACACA 219
pMC1F-1   > ATCTCGGCGGCCAGACGATGGTCGTAAGGGGAGCATCGTCTCCGTCACGACAGTAACACA 352
pMC1F-2   > ATCTCGGCGGCCAGACGATGGTCGTAAGGGGAGCATCGTCTCCGTCACGACAGTAACACA 352
MC2-MC5P2 < ATCTCGGCGGCCAGACGATGGTCGTAAGGGGAGCATCGTCTCCGTCACGACAGTAACACA 387
CONSENSUS > ATCTCGGCGgCCAGACGATGgTCGTAAGGGGAGCATCGTCTCCGTCACGACAGTAACACA 1080

          .........+.........+.........+.........+.........+.........+

pMC1R-2   <                                 CTGACCAAAAGCATGACTGGTGGGGATGAGGT 32
pMC1P2-1  < GCAATACGAACCCGTCAACTCTCCCCgACTGACnAAAAGCATnACTGGTGGGGATnAGGT 159
pMC1P2-2  < GCAATACGAACCCGTCAACTCTCCCCGACTGACCAAAAGCATGAC                264
pMC1F-1   > GCAATACGAACCCGTCAACTCTCCCCGACTgaccaaaagcatgactggtggggatgaggt 412
pMC1F-2   > GCAATACGAACCCGTCAACTCTCCCCGACTGACcAAAAGCATGACTGGTGGGGATGAGGT 412
MC2-MC5P2 < GCAATACGAACCCGTCAACTCTCCCCGACTGACcAAAAGCATgACTGGTGGGGATGAGGT 447
CONSENSUS > GCAATACGAACCCGTCAACTCTCCCCGACTGACcAAAAGCATgACTGGTGGGGATgAGGT 1140

          .........+.........+.........+.........+.........+.........+

pMC1R-1   <      TTGCTGGCATCGAGGAGGCACCTATGGcGCGAGGTCTACTCATTCTGGcGCAGAT 55
pMC1R-2   < TCTGTTTGCTGGCATCGAGGAGGCACCTATGGCGCGAGGTCTACTCATTCTGGCGCAGAT 92
pMC1P2-1  < TCTGTTtGCTGGCATCgAGgAgGCACCTAT                               189
pMC1F-1   > tctgtttgctggcatcgaggaggcacctatg                              443
pMC1F-2   > TCTGTTTGCTGGCATCGAGGAGGCACCTATGGCGCGAGGTCTACTCATTCTGGCGCAGAT 472
MC2-MC5P2 < TCTGTTtGCTGGC                                                460
CONSENSUS > TCTGTTTGCTGGCATCGAGGAGGCACCTATGGCGCGAGGTCTACTCATTCTGGCGCAGAT 1200

          .........+.........+.........+.........+.........+.........+

MC1-MC3P3 >                                 TACACAAgCtTGCgTGGnGGCTGCCAG 27
pMC1P3-1  >                       TGAnTaAGGnGTATACACAAGCtTGCgTGGAGGCTGCCAG 40
pMC1R-1   < GTCCTCGGCGGGGAACTTTATGAATAAGGagTATACACAAGCTTGCGTGGAGGCTGCCAG 115
pMC1R-2   < GTCCTCGGCGGGGAACTTTATGAATAAGGAGTATACACAAGCTTGCGTGGAGGCTGCCAG 152
pMC1F-2   > GTCCTCGGCGGGGAACTTTATGAATAAGGAGTATACACA                     511
CONSENSUS > GTCCTCGGCGGGGAACTTTATGAaTAAGGaGTATACACAAGCTTGCGTGGaGGCTGCCAG 1260

          .........+.........+.........+.........+.........+.........+

MC1-MC3P3 > AGAACACAAAGACTTTgTaATGGGATTTgTATCACAAGAgACGTTGAATACGGAACTCGA 87
pMC1P3-1  > AgAACACAAAGACTTTGTAATGGGATTTGTATCACAAGAgACGTTGAATACGGAACTCGA 100
pMC1R-1   < AGAAcACAAAGACTTTGTAATGGgaTTTGTATCACAAGAGACGTTGAATACGGaACTCGA 175
pMC1R-2   < AGAACACAAAGACTTTGTAATGGGATTTGTATCACAAGAGACGTTGAATACGGAACTCGA 212
CONSENSUS > AGAACACAAAGACTTTGTAATGGGATTTGTATCACAAGAGACGTTGAATACGGAACTCGA 1320

          .........+.........+.........+.........+.........+.........+
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MC1-MC3P3 > TGACGAATTTaTTCACATGACGCCAGGCTGCCAGCTGCCGCCCGAaGACGAGGATCAAAA 147
pMC1P3-1  > TGACGAATTTATTCACATGACGCCAGGCTGCCAGCTGCCGCCCGAaGACGAGGATCAAAA 160
pMC1R-1   < TGACGAATTTATTCacATGACGCCAgGCTGCCAGCTGCCGcCCGAAgACGAGGaTCAAAA 235
pMC1R-2   < TGACGAATTTATTCACATGACGCCAGGCTGCCAGCTGCCGCCCGAAGACGAGGATCAAAA 272
CONSENSUS > TGACGAATTTATTCACATGACGCCAGGCTGCCAGCTGCCGCCCGAAGACGAGGATCAAAA 1380

            .........+.........+.........+.........+.........+.........+

MC1-MC3P1 <                                  CAGTATAATACACCACAAAAGATTATCGG 29
MC1-MC3P3 > CGGCACAGTCCAGGGCGACGGAAAAGGCCAGCAGTATAATACACCACAAAAGATTATCGG 207
pMC1P3-1  > CGGCACAGTCCAGGGCGACGGAAAAGGCCAGCAGTATAATACACCACAAAAgATTATCGG 220
pMC1R-1   < CGGcacAGTCCAGGgCGACGnnAAAGnCCAGCaGTATAATACACcACAAAAgATTATCGG 295
pMC1R-2   < CGGCACAGTCCAGGGCGACGGaAAAGGCCAGCAGTATAATACACCACAAAAGATTATCGG 332
CONSENSUS > CGGCACAGTCCAGGGCGACGgaAAAGgCCAGCAGTATAATACACCACAAAAGATTATCGG 1440

            .........+.........+.........+.........+.........+.........+

pMC4R-2   >                                              AAAGCAGGGGACCCCGA 17
pMC4P1-3  <                                  GGATCCTCAAAGCAGGGGACCCCGA 25
pMC4P1-2  <                                  GGATCCTCAAaGCAGGGGACCCCGA 25
pMC4P1-1  <                                  GGATCCTCAAaGCAGGGGACCCCGA 25
MC1-MC3P1 < CGTAGCTGGAGCGGACATCGTCATTGTCGGCCGTGGGATCCTCAAAGCAGGGGACCCCGA 89
MC1-MC3P3 > CGTAGCTGGAGCGGACATCGTCATTGTCGGCCGTGGGATCCTCAAAGCAGGGGACCCCGA 267
pMC1P3-1  > CGTAgcTGGAGCgGACATCGTCATTGTCGGCCGTGGGATCC                     261
pMC1R-1   < cGTAGcTGGaGCGGACATCGTCATTGTCGgCCGTGGGATCC                     336
pMC1R-2   < CGTAGCTGGAGCGGACATCGTCATTGnCGGCCG                             365
CONSENSUS > CGTAGCTGGAGCGGACATCGTCATTGtCGGCCGTGGGATCCTCAAAGCAGGGGACCCCGA 1500

            .........+.........+.........+.........+.........+.........+

pMC4R-2   > GGGCGAAACGgAGCGATATCGCTCTGcGGCTTGGAAGGGTTACACTGAGAGGGTTCGCTA 77
pMC4P1-3  < GGGCGAAACGGAGCGATATCGCTCTGCGGCTTGGAAGGGTTACACTGAGAGGGTTCGCTA 85
pMC4P1-2  < GGGCGAnACGGAGCGATATCGCTCTGCGGCTTGGAAGGGTTACACTGAGAGGGTTcGCTA 85
pMC4P1-1  < GGGCGAAACGGAGCGATATCGCTCTGCGGCTTGGAAGGGTTACACTGAGAGGGTTCGCTA 85
MC1-MC3P1 < GGGCGAaACGGAGCGATATCGCTCTGCGGCTTGGAAGGGTTACACTGAGAGGGTTCGCTA 149
MC1-MC3P3 > GGGCGAAACGGAGCGATATCGCTCTGCgGCTTGGAAGGGTTACACTGAGAGGGTTCGCTA 327
CONSENSUS > GGGCGAaACGGAGCGATATCGCTCTGCGGCTTGGAAGGGTTACACTGAGAGGGTTCGCTA 1560

            .........+.........+.........+.........+.........+.........+

pMC4R-2   > GCAACTTGAGCCAGGTGGCTGGTCATTGGTTCTgAaAATnttnCttGAnACTaACCTGAC 137
pMC4P1-3  < GCAACTTGAGCCAGGTGGCTGGTCATTGGTTCTGAaAATTT                     126
pMC4P1-2  < GCnACtTGAGCCAGGTGGCTGGTCATTGGTTCTnnnnnTTTTGCTTGAAACTAACCTGAC 145
pMC4P1-1  < GCAACTTGAGCCAGGTGGCTGGTCATTGGTTCTGAAAATTTTGCTTGAAACTAACCTGAC 145
MC1-MC3P1 < GCAACTTGAGCCAGGTGGCTGGTCATTGGTTCTGAAAATTTTGCTTGAAACTAACCTGAC 209
MC1-MC3P3 > GCAACTTGAGCCAGGTGGCTGGTCATTGGTTCTG                           361
CONSENSUS > GCaACTTGAGCCAGGTGGCTGGTCATTGGTTCTgaaaaTtTTgCTTGAaACTAACCTGAC 1620

            .........+.........+.........+.........+.........+.........+

pMC4R-2   > GTTTTGAAGGAAGAAGCACAAACATGGGAAAaATAGGAGGTACGCATCATTCTCCGTtTC 197
pMC4P1-2  < GTTTTGAAGgaAGAAGCACAnACATGGnnnAaATAGgAGGTACGCATCATTCTCCGTTTC 205
pMC4P1-1  < GTTTTGAAGGAAGAAGcACAaACATGGgaaAAATAGGAGGTACGCATCATTCTCCGTTTC 205
MC1-MC3P1 < GTTTTGAAGGAAGAAGCACAAACATGGgaaAAATAGGAGGTACGCATCATTCTCCGTTTC 269
CONSENSUS > GTTTTGAAGGAAGAAGCACAaACATGGgaaAAATAGGAGGTACGCATCATTCTCCGTTTC 1680

            .........+.........+.........+.........+.........+.........+

pMC4R-2   > GGCATCATGTGTTACGCTTAGCTAATTATGGAATACAAGCATCCTTCCCCCCAATTCTCT 257
pMC4P1-2  < GGCATCATnTGTTACGCTTAGCTAATTATGGAAnACAAGCATCCTTCCCCCCAATTCTCT 265
pMC4P1-1  < GGCATCATgTGTTAcGCTTAGCtAATTATGGAAtACAAGCATCCTTCCCcCCAAtTctct 265
MC1-MC3P1 < GGCATCATGTGTTACGCTTAGCTAATTATGGAAtACAAGCATCCTTCCCCCCAATTCTC  328
CONSENSUS > GGCATCATgTGTTACGCTTAGCTAATTATGGAAtACAAGCATCCTTCCCCCCAATTCTCT 1740

            .........+.........+.........+.........+.........+.........+
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pMC4R-2    > TTGACAACAATCAACTTCCTAACCCGTAATGGCATTAGGCTTTGAAGAAAATtGGAttCG 317
pMC4P1-2   < TTnAC                                                        270
pMC4P1-1   < ttGAc                                                        270
CONSENSUS  > TTgACAACAATCAACTTCCTAACCCGTAATGGCATTAGGCTTTGAAGAAAATTGGATTCG 1800

             .........+.........+.........+.........+.........+.........+
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APPENDIX 4.0 Published paper

Schardl, C. L., A. Leuchtmann, H.-F. Tsai, M. A. Collett, D. M. Watt and D. B. Scott (1994). Origin of a fungal symbiont of perennial ryegrass by interspecific hybridization of a mutualist with the ryegrass choke pathogen, *Epichloë typhina*. **Genetics 136**: 1307-1317.

Origin of a Fungal Symbiont of Perennial Ryegrass by Interspecific Hybridization of a Mutualist with the Ryegrass Choke Pathogen, *Epichloë typhina*

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ABSTRACT

Seed-borne fungal symbionts (endophytes) provide many cool-season grass species with biological protection from biotic and abiotic stresses. The endophytes are asexual, whereas closely related sexual species of genus Epichloë (Clavicipitales) cause grass choke disease. Perennial ryegrass (*Lolium perenne*) is a host of two endophyte taxa, LpTG-1 (*L. perenne* endophyte taxonomic grouping one = *Acremonium lolii*) and LpTG-2, as well as the choke pathogen, *Epichloë typhina* (represented by isolate E8). Relationships among these fungi and other Epichloë species were investigated by analysis of gene sequences, DNA polymorphisms and allozymes. The results indicate that LpTG-2 is a heteroploid derived from an interspecific hybrid. The LpTG-2 isolates had two copies each of nine out of ten genes analyzed (the exception being the rRNA gene locus), and the profiles for seven of these were composites of those from *E. typhina* E8 and *A. lolii* isolate Lp5. Molecular phylogenetic analysis grouped the two β-tubulin genes of LpTG-2 into separate clades. One (*tub2-1*) was related to that of *E. typhina* E8, and the other (*tub2-2*) to that of *A. lolii*. The mitochondrial DNA profile of LpTG-2 was similar to that of *A. lolii*, but its rRNA gene sequence grouped it with *E. typhina* E8. A proposed model for the evolution of LpTG-2 involves infection of a *L. perenne-A. lolii* symbiotum by *E. typhina*, followed by hybridization of the two fungi. Such interspecific hybridization may be a common and important mechanism for genetic variation in Epichloë endophytes.

ASSOCIATIONS of grasses with fungal species of genus *Epichloë* (Clavicipitales) and related asexual endophytes (classified in section *Albo-lanosa* of form genus *Acremonium* of the Fungi Imperfecti) comprise an evolutionary continuum from mutualism to antagonism. Among the associations of grasses with Epichloë species, the degree of mutualism or antagonism is related to the relative importance of the asexual or sexual life cycle of the fungus (CLAY 1988). Asexual endophytes cause no disease and are apparently disseminated only by clonal propagation in host seed. Where fitness of the hosts has been assessed, the endophytes have conferred demonstrable benefits including protection from herbivory (mammalian and insect), disease (fungal and nematode) and drought, and increased production of biomass and seeds (CLAY 1990; KIMMONS *et al.* 1990; RICE *et al.* 1990). In contrast, the Epichloë species in their sexual life cycle, by suppressing host seed production, can be antagonistic to the life cycles of both the hosts and the asexual endophytes. The similarities of their endophytic growth habits, morphological characteristics and secondary metabolites (SIEGEL *et al.* 1990; WHITE *et al.* 1991) suggest a close evolutionary relationship between sexual Epichloë species and asexual endophytes. These relationships have been confirmed by molecular genetic analyses based on allozyme profiles (CHRISTENSEN *et al.* 1993; LEUCHTMANN and CLAY 1990) and DNA sequence comparisons (AN *et al.* 1992; SCHARDL *et al.* 1991), which support two significant conclusions. First, there appear to have been multiple evolutionary origins of the asexual endophytes from Epichloë species. Second, even endophyte isolates from the same grass species can show considerable genetic diversity.

Epichloë species and their asexual relatives persist and grow endophytically without causing disease in vegetative tissues of their hosts (BACON and DE BATTISTA 1990) and are closely associated with meristematic tissues that give rise to leaf sheaths, tillers, rhizomes and inflorescences. The endophytes and many Epichloë species can infect host seed, also without discernible detriment to the host. As grass inflorescences develop, endophytic hyphae grow into the ovules and ultimately infect the endosperms and embryos in the seeds (PHILIPSON and CHRISTEY 1986). Infected seed is very likely the only means of propagation of the asexual endophytes. Thus, clonal endophyte lineages are associated with the maternal lineages of their hosts. Such an extreme linkage by common descent should select for enhanced mutualism during evolution (SIEGEL and SCHARDL 1991).

The sexual cycles of pathogenic Epichloë species are

TABLE 1

Fungal isolates in this study and their characteristics

Species or taxon	Isolate	Mating type	Host species	Geographic origin of host	Sexual expression of fungus	Reference, cultivar or source
LpTG-2	Lp1	mat-2	*Lolium perenne*	S. France	None	Christensen *et al.* (1993)
LpTG-2	Lp2	Unknown	*L. perenne*	S. France	None	Christensen *et al.* (1993)
Acremonium lolii (=LpTG-1)	Lp5	Unknown	*L. perenne*	Unknown	None	cv. Nui
A. lolii	Lp9	Unknown	*L. perenne*	S. France	None	Christensen *et al.* (1993)
A. lolii	e44	Unknown	*L. perenne*	Unknown	None	cv. Repel
A. lolii	e46	Unknown	*L. perenne*	Unknown	None	cv. Chantely
A. lolii	Lp14	Unknown	*L. perenne*	S. France	None	Christensen *et al.* (1993)
Epichloe typhina (=MP-I)	E8	mat-1	*L. perenne*	Europe	High	Byrd *et al.* (1990)
E. typhina	E2461	mat-2	*Dactylis glomerata*	Britain	High	White (1993)
E. typhina	E2466	mat-2	*D. glomerata*	Switzerland	High	D. Schmidt
Epichloe sp. MP-II	E28	mat-2	*Festuca longifolia*	Europe	Low	cv. SR3000
Epichloe sp. MP-II	E32	mat-1	*Festuca rubra* subsp. *commutata*	Europe	Low	cv. Longfellow
Epichloe sp. MP-II	E189	mat-2	*F. rubra* subsp. *rubra*	Europe	Low	Schardl and Tsai (1992)
Epichloe sp. MP-III	E56	mat-1	*Elymus canadensis*	Texas	Low	White and Bultman (1987)
Epichloe sp. MP-IV	E52	mat-2	*Sphenopholis obtusata*	Georgia	High	Bacon and Hinton (1991)
Epichloe sp.	E57	Unknown	*Agrostis hiemalis*	Texas	Low	White *et al.* (1991)
Epichloe sp.	E248	mat-1	*Agrostis stolonifera*	Britain	High	White (1993)

initiated by the production of stromata, sporogenous mycelial structures on host leaf sheaths and inflorescences that prevent maturation of the florets and thereby suppress seed production on the affected tillers (choke disease). The fungal stromata give rise to asexual spores (conidiospores) that serve as spermatia in mating (White and Bultman 1987). The sexual cycle culminates in the discharge of haploid ascospores derived from the meiotic products. In extreme cases of antagonism the hosts are completely sterilized. However, many Epichloë species are pleiotropic symbionts (Michalakis *et al.* 1992) that can exhibit both the sexual life cycle of antagonists and the asexual life cycle of the mutualists. In these cases, some flowering tillers of an infected plant may exhibit chokes, whereas others on the same plant are unaffected, giving rise to seeds containing the fungus (Saha *et al.* 1987).

Perennial ryegrass is a known host of three species or taxa in the Epichloë group. The two known taxa of asexual, seed-borne endophytes are *Acremonium lolii* (Latch *et al.* 1984)–also classified as taxonomic grouping one from *Lolium perenne* (LpTG-1)–and LpTG-2 (Christensen *et al.* 1993). Benefits to perennial ryegrass, particularly of *A. lolii* mutualism, have been extensively documented (Clay 1990). In contrast, *Epichloë typhina* (*i.e.*, Epichloë mating population I = MP-I) represents the antagonistic extreme, completely sterilizing infected perennial ryegrass plants (Siegel *et al.* 1987). In this report, the genetic compositions and evolutionary relationships of the perennial ryegrass endophytes and *E. typhina* are explored, and the results indicate that LpTG-2 is a heteroploid derived from a hybrid whose most likely ancestors are *A. lolii* and *E. typhina*.

MATERIALS AND METHODS

Biological materials: Fungal isolates and their hosts and characteristics are listed in Table 1. Methods for their isolation from infected plants, culture, maintenance and characterization by serology are described elsewhere (An *et al.* 1993; Schardl and An 1993). Single conidiospore isolation of LpTG-2 isolate Lp1 was performed three times in succession. Each time, conidiospores were streaked, then monitored daily by microscope (50 ×) to ensure that colonies were chosen from germinated conidiospores and not from hyphal fragments. The method of Wilson (1992) was used to stain nuclei of conidiospores.

To identify biological species (*i.e.*, mating populations or MP) of the Epichloë isolates, mating tests were carried out as described previously (Schardl and Tsai 1992; White 1993). Epichloë completes its sexual cycle only after mating between individuals of the same biological species and of opposite mating type (*mat-1* and *mat-2*). For example, isolate E8 from perennial ryegrass was identified as *E. typhina sensu stricto* because it successfully mated with a known *E. typhina* isolate (designated E2461 in Table 1) from another grass species (White 1993). Test matings also indicated that isolates E32, E28 and E189, all from Festuca species grasses, belong to a second mating population, MP-II, and can not be mated with E8, E2461, E2466, E52, E56 or E248.

DNA isolation: Total fungal DNA was prepared as previously described (Byrd *et al.* 1990; Murray *et al.* 1992). Mitochondrial DNA (mtDNA) was isolated as described by Mogen *et al.* (1991) with modifications. Isolate Lp1 was grown for 1 week on five 135-mm diameter cellophane disks on potato-dextrose agar (Difco, Detroit, Michigan) (Schardl and An 1993) and ground in cold buffer containing 15% sucrose, 10 mM Tricine-KOH pH 7.5, 0.2 mM ethylenediaminetetraacetic acid (EDTA).

The cellular debris and nuclei were removed by centrifugation at 1,000 × *g* for 10 min, then reextracted once. The supernatants were pooled and centrifuged at 15,000 × *g* for 15 min, then the pelleted mitochondria were resuspended in 20% sucrose, 10 mM Tricine-KOH, pH 7.5, 0.1 mM EDTA and repelleted. Mitochondria were resuspended in buffer C [1.75 M sucrose, 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5, 5 mM EDTA], brought to 12 mM $MgCl_2$ and 100 μg ml^{-1} deoxyribonuclease I, incubated 90 min at 4°, repelleted at 20,000 × *g* for 10 min, washed in buffer C, and lysed in 0.44 M sucrose, 1% sodium dodecyl sulfate (SDS). Proteinase K (Sigma Chemical Co., St. Louis, Missouri) was added to 55 μg ml^{-1}, and the extract was incubated 45 min at 37°, then extracted with Tris-HCl-saturated phenol (pH 8.0), phenol:chloroform:isoamyl alcohol (25:24:1), and chloroform:isoamyl alcohol (24:1). The DNA was concentrated by centrifugal dialysis in Centricon-30 cells (Amicon Div., Beverly, Massachusetts), precipitated by addition of sodium acetate to 300 mM and two volumes of ethanol, rinsed in 70% ethanol, and redissolved in 10 mM Tris-HCl pH 8, 1 mM EDTA.

DNA amplification and sequence determination: Most oligonucleotide primers for amplification and for sequence analysis were described previously (Byrd *et al.* 1990; Schardl *et al.* 1991). The 5′ portions of *tub2*, inclusive of introns IVS1, IVS2 and IVS3, were amplified using primers 1042 (5′-GAGAAAATGCGTGAGATTGT-3′) and 1214 (5′-TGGTCAACCAGCTCAGCACC-3′), homologous to conserved, protein coding sequences. In some cases the 5′ segments of *tub2* genes could be amplified using primer 4414 (5′-CTGGTGCCTGAGATACCGC-3′) in place of primer 1042. Primer 4414 was homologous to sequence approximately 320 bp upstream of the start codon of some, but not all, *tub2* genes in the study. Amplification of *rrn* ITS1 and ITS2 regions (the internal transcribed spacers of the rRNA genes) employed primers described by White *et al.* (1990). Polymerase chain reactions (PCR) were performed as described previously (Schardl *et al.* 1991) using either *Taq* DNA polymerase (Boehringer-Mannheim Biochemical, Indianapolis, Indiana) or AmpliTaq™ (U.S. Biochemical Corp., Cleveland, Ohio). A Perkin-Elmer (Norwalk, Connecticut) thermal cycler was programmed for the following temperature regimes: one incubation at 94° for 105 sec; 35 cycles of 94° 45 sec, 55° 45 sec, 72° 75 sec; one 5-min incubation at 72°; then cooling to 4°. Products were purified using Magic Prep™ resin cartridges (Promega Corp., Madison, Wisconsin).

Sequences were obtained directly from both strands of each PCR product using the fmol™ kit from Promega Corp. The purpose for sequencing the PCR products directly, rather than cloning them first, was to virtually eliminate the potential for sequencing artifacts due to occasional misincorporations by Taq DNA polymerase (Thomas and Kocher 1993). Reactions were as recommended by the manufacturer, with modifications. Each of four termination reactions (6 μl) contained 50 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 200 nM sequencing primer (Byrd *et al.* 1990; White *et al.* 1990), 40–200 fmol template DNA, 0.2 unit μl^{-1} modified Taq DNA polymerase (sequencing grade from Promega), 2 μCi [α-^{35}S]dATP (>1000 Ci $mmol^{-1}$; DuPont-NEN, Boston, Masschusetts), 6.7 μM each of dTTP, dCTP, dATP and 7-deaza dGTP, plus a dideoxynucleotide triphosphate (10 μM ddGTP, 117 μM ddATP, 200 μM ddTTP or 67 μM ddCTP). The reactions were performed with 30 temperature cycles of 95° 30 sec, 58° 30 sec, 70° 30 sec. The products analyzed by acrylamide gel electrophoresis (Ausubel *et al.*, 1987–1993).

Sequences have been deposited in GenBank and EMBL databases under accession numbers L06956, L06958, L07130–L07133, L07138, L07139, L07141, L07142, L06955, L06957 and L06959–L06962.

Phylogenetic analysis: Because of the close relationships among the sequences, all alignments were made by eye.

Most parsimonious trees were inferred from the aligned sequences using the branch-and-bound search, implemented in PAUP (Swofford 1993), to obtain exact solutions. Nucleotide substitutions were unordered and unweighted. Alignment gaps were either treated as missing information or recoded for parsimony. In the latter case, only gaps identical in all sequences possessing them were recoded, and each recoded gap was treated as one nucleotide substitution regardless of its length (see Figures 1 and 3). Structures of the trees were similar whether or not gaps were recoded, though there was a modest increase in bootstrap support of some branches. Bootstrap analysis (Felsenstein 1985) used 100 replications by branch-and-bound.

Neighbor-joining (Saitou and Nei 1987) trees were calculated using PHYLLIP (Felsenstein 1993). Alignment gaps were treated as missing information. The tree structures were the same whether one or two-parameter distance corrections were employed.

Analysis of DNA polymorphisms: Restriction endonuclease digestion, electrophoretic separation and Southern transfers were as previously described (Murray *et al.* 1992; Tsai *et al.* 1992). For detection of *pyr4* sequences a ^{32}P-labeled probe was generated from the 0.65-kb *Stu*I fragment of clone pRS4 (Smit and Tudzynski 1992). This probe includes the *pyr4* gene of *Claviceps purpurea*. DNA hybridizations were carried out at 65° in 3 × SSC (Whitfeld *et al.* 1982), and washes were at 65° first in 3 × SSC and finally in 1 × SSC containing 0.1% SDS. Clones of *pyr4* genes were from an EMBL λ phage library prepared and screened by standard protocols (Sambrook *et al.* 1989).

For detection of *tub2* sequences a digoxigenin-labeled probe was generated by PCR (Lion and Haas 1990) using as template a cloned *tub2* gene from the tall fescue endophyte FaTG-1 isolate e19 (Tsai *et al.* 1994). The probe was 980 bp extending from codon 1–199 of the gene, and including the first three introns. Hybridization, wash and detection procedures were as previously described (Tsai *et al.* 1992).

Allozymes: Allozyme analyses were by horizontal starch gel electrophoresis, as described previously (Christensen *et al.* 1993; Leuchtmann and Clay 1990).

RESULTS

Detection of two β-tubulin genes in LpTG-2 isolates: Sequences of the 5′ regions of *tub2* genes, including introns IVS1, IVS2 and IVS3, are aligned in Figure 1. All sequence determinations were initially attempted by analyzing products of PCR reactions that employed genomic DNA templates and primers homologous to the conserved coding regions. In nearly all cases the sequences were unambiguous, indicating only a single form of the gene in most isolates. The only exceptions were the *tub2* sequences of the LpTG-2 isolates.

After the *tub2* 5′ regions were amplified from isolates Lp1 and Lp2, numerous ambiguities in the sequences of the PCR products suggested that these isolates had two *tub2* genes. These ambiguities were observed only with products amplified using primers 1042 (exon 1) and 1214 (exon 4). When an upstream primer, 4414, was used in place of primer 1042 only one sequence was

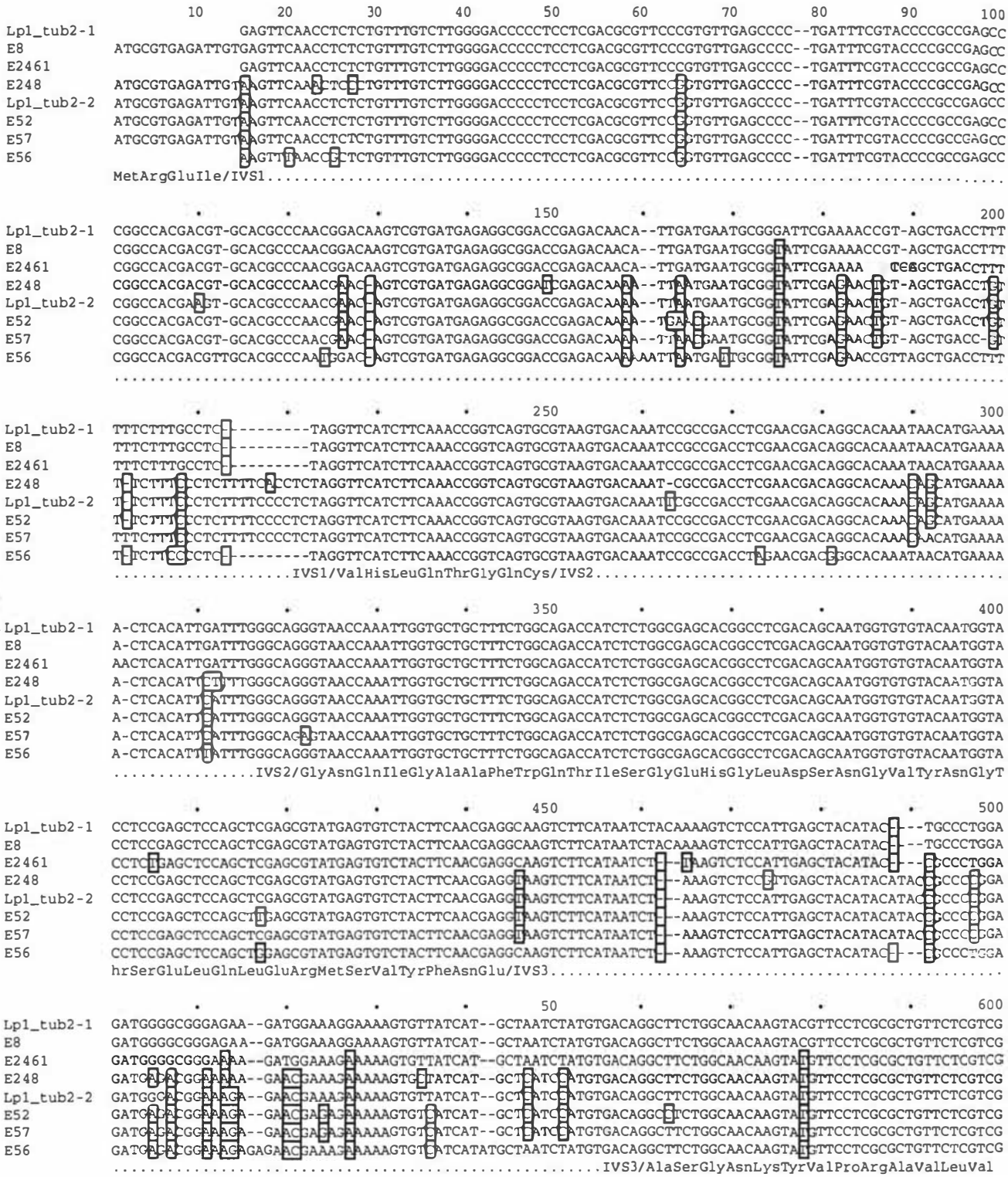

FIGURE 1.—Sequence alignment of *tub2* 5′ regions. The Lp1 *tub2–1* sequence shown here was identical to that of the other LpTG-2 isolate, Lp2. Also, the Lp1 *tub2-2* sequence was identical to those of Lp2, Lp5, E28, E32 and E189. Dashes (–) indicate alignment gaps, and those dashes enclosed in boxes indicate alignment gaps sometimes recoded as characters in parsimony analysis.

obtained from the LpTG-2 isolates. This sequence was identical to that of *A. lolii* isolate Lp5, and to those of the Epichloë MP-II isolates, and the gene copy from which it was derived was designated *tub2-2*. An *Ase*I recognition site was present in a variable region (positions 159–166 in Fig. 1), so in order to prevent amplification of *tub2-2*, Lp1 and Lp2 genomic DNAs were first digested with *Ase*I and subsequently used as templates in

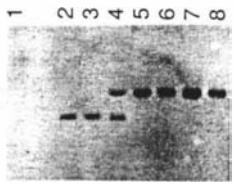

FIGURE 2.—Identification of two *tub2* genes in LpTG-2 isolate Lp1. Mitochondrial DNA (lane 1) was from LpTG-2 isolate Lp1 (lane 1), and total DNA was from *E. typhina* isolates E2466 (lane 2), and E8 (lane 3), LpTG-2 isolate Lp1 (lane 4), *A. lolii* isolates e44 (lane 5) and e46 (lane 6), and Epichloë MP-II isolates E32 (lane 7) and E189 (lane 8). The DNA samples were cleaved with *Bam*HI and *Pst*I, electrophoresed in 0.7% agarose, blotted and probed with an amplified fragment from the 5′ region of *tub2*. Sizes of the hybridizing fragments are indicated in kilobasepairs (kb). The two *tub2* genes in Lp1 (lane 4) gave rise to fragments of sizes 1.7 kb (*tub2-1*) and 2.2 kb (*tub2-2*).

PCR reactions with primers 1042 and 1214. Sequences of the amplification products obtained in this manner were unambiguous, and nearly identical to the sequence from *E. typhina* isolate E8 (Figure 1). Thus, a second gene copy, designated *tub2-1*, was identified in the two LpTG-2 isolates.

The presence of two *tub2* copies in isolate Lp1 was confirmed by analysis of total DNA, cleaved with *Pst*I and *Bam*HI and hybridized to a 980-bp digoxigenin-labeled probe which included the first three introns and codons 1–19 of *tub2* (Figure 2). Cleavage of genomic DNA from *E. typhina* E8 (lane 3) released a 1.7-kb hybridizing fragment extending from 235 bp upstream of the start codon to a *Bam*HI site at codons 344–346 (BYRD *et al.* 1990). A fragment of similar size was obtained from the other *E. typhina* isolates E2466 (lane 2) and E2461 (H.-F. TSAI unpublished data). However, a larger fragment (2.2 kb) was obtained from *A. lolii* and from Epichloë MP-II isolates E32 and E189 (lanes 5–8), which lacked the *Pst*I site at position −235 bp. The LpTG-2 isolate Lp1 (lane 4) had *tub2*-homologous fragments of both sizes, the larger arising from *tub2-2* and the smaller from *tub2-1*.

Phylogenetic analysis of rRNA and β-tubulin genes: To determine the origin of the two *tub2* genes in LpTG-2 a phylogenetic analysis was conducted on sequences of the *tub2* introns, as well as the *rrn* ITS1 and ITS2 regions. The sequences of ITS1 and ITS2 are aligned in Figure 3. As in the analysis of *tub2*, PCR amplification and subsequent sequence analysis of the *rrn* segments were performed to identify ambiguities, but these were rarely observed. The only ambiguity detected, at position 93 (Figure 3) of ITS1 from E2461, was confirmed by analysis of both DNA strands. This indicated a polymorphism possibly due either to a mixture of two related genotypes (E2461 was not single-spore isolated) or to variation among the tandem rRNA gene copies [the typical arrangement in *rrn* loci in fungi (FREE *et al.* 1979)]. The ambiguity represented only a minor difference not affecting phylogenetic inferences. Only a single sequence of this region was detected in the LpTG-2 isolates; this was identical to ITS1 and ITS2 of *E. typhina* E8. Southern blot analysis of the rRNA structural gene also identified only a single type in isolate Lp1, similar to that of *E. typhina* E8, but distinct from those of *A. lolii*, Epichloë MP-II and other *E. typhina* isolates (H.-F. TSAI and D. M. WATT unpublished data).

The phylogenetic relationships of the *rrn* and *tub2* sequences were inferred by parsimony and by the distance-based method, neighbor-joining (Figure 4). The known sexual (Epichloë) isolates showed convergence of the *tub2* and *rrn* phylogenies, strongly suggesting that the gene trees accurately reflected the evolutionary relationships of the organisms (PENNY *et al.* 1982) and that most of the observed sequence divergence occurred after speciation. Furthermore, both trees linked the *A. lolii* isolate Lp5 with the isolates of Epichloë MP-II.

The sequences of the two *tub2* copies in the LpTG-2 endophytes were related much more closely to the single *tub2* genes of other isolates than to each other (Figure 4). The *tub2-1* and *tub2-2* sequences differed by 54% of the variable sites (28 nucleotide substitutions). In contrast, *tub2-1* differed from *tub2* of *E. typhina* E8 by only one substitution (2%), and the *tub2-2* sequence was identical to that of *A. lolii* isolate Lp5 and the Epichloë MP-II isolates. Bootstrap analysis strongly supported the separation of *tub2-1* and *tub2-2* into separate clades. Hence, phylogenetic inference for the *tub2* copies strongly suggests gene transfer or interspecific hybridization in the evolution of the endophyte taxon LpTG-2.

There was only one discernible *rrn* form in the LpTG-2 isolates, and their ITS1 and ITS2 sequences were identical to those of *E. typhina* E8. Therefore, the *rrn* phylogeny of the LpTG-2 isolates converged with the *tub2-1* phylogeny.

Isozymes and pyr4 polymorphisms: Two *pyr4* genes were cloned from the Lp1 genome (Figure 5). Following *Bam*HI digestion one of the genes, *pyr4-1* (lane 1), appeared similar to that of *E. typhina* E8 (lane 2); the other, *pyr4-2* (lane 6), appeared similar to those of *A. lolii* isolates Lp5 and Lp9 (lanes 4 and 5).

Allozyme profiles (Table 2) also indicated multiple gene copies in the LpTG-2 isolates. Seven isozyme loci were identified which, because they exhibited variation among *A. lolii* and *E. typhina* isolates, could be used to test the hypothesis that LpTG-2 had a hybrid origin. For each locus, two allozymes were observed from Lp1 and Lp2. Five of the seven patterns could be explained as combinations of allozyme profiles that typify *E. typhina* isolate E8 and *A. lolii* isolate Lp5. In the cases of phosphoglucose isomerase and phosphoglucomutase-1, one allozyme could have been derived from the E8 genotype, but the origin of the other was not apparent. Possibly the specific ancestral *A. lolii*

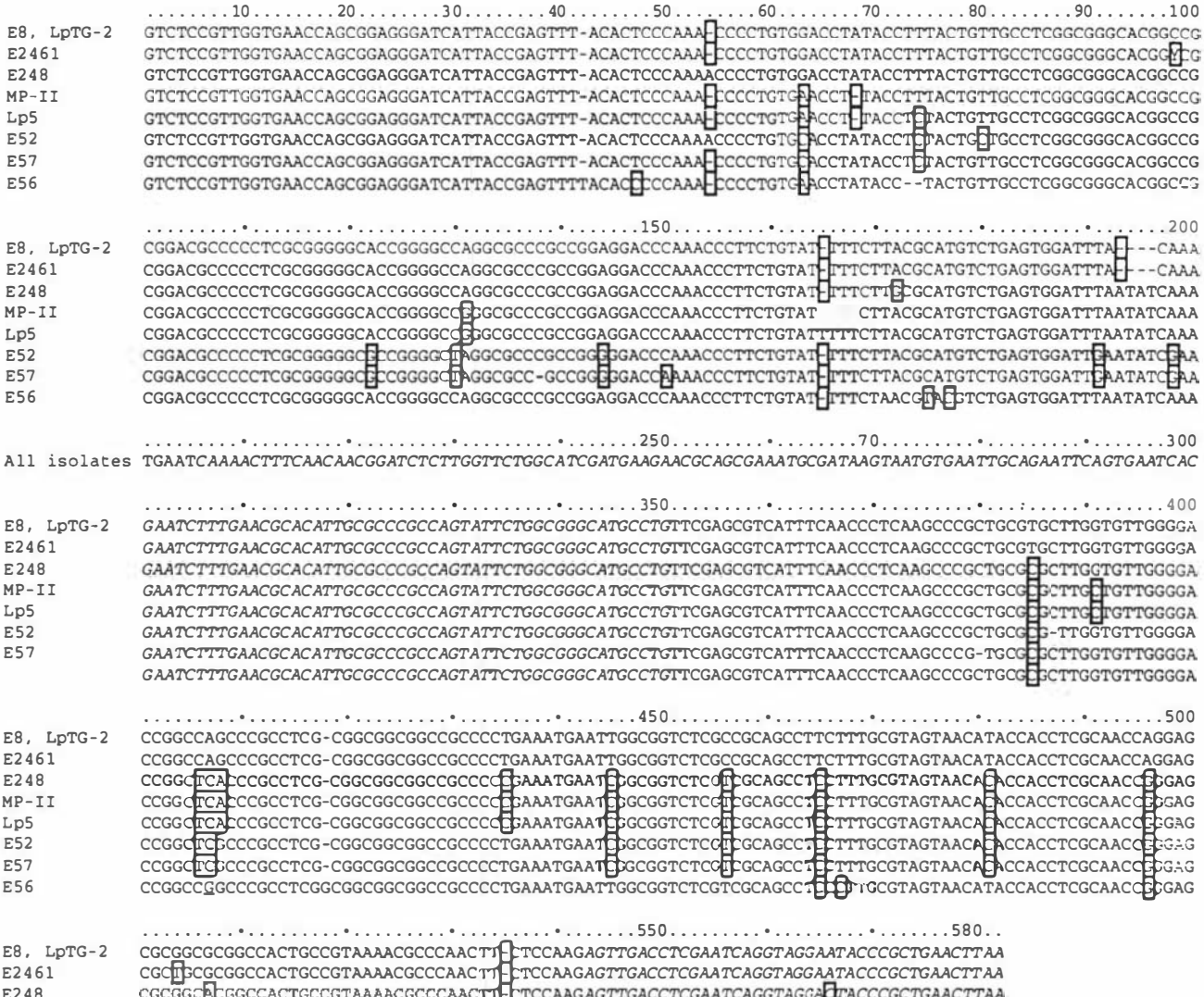

FIGURE 3.—Sequence alignment of *rrn* gene segments including ITS1 (positions 1–205) and ITS2 (positions 351–542). Sequences listed as LpTG-2 were from isolates Lp1 and Lp2. Sequences listed as MP-II were from isolates E28, E32 and E189. The sequences from isolates E8, Lp5 (=e2), E32 and E56 were reported earlier (SCHARDL *et al.* 1991). Hyphens enclosed in boxes indicate the alignment gaps recoded as equivalent to a nucleotide substitution for the purpose of parsimony analysis.

genotype was not among those sampled [Table 2; and see CHRISTENSEN *et al.* (1993)]. Alternatively, mutations affecting allozyme mobility may have occurred in LpTG-2 after hybridization.

Mitochondrial DNA profiles: Due to their high copy number relative to single-copy nuclear DNA, mtDNA fragments from restriction endonuclease-digested total DNAs were observed as intense bands in electrophoretograms (Figure 6). The mtDNA profile from Lp1 exactly matched that of *A. lolii* isolates e44 and e46, but differed substantially from those of *E. typhina* and Epichloë MP-II isolates (Figure 6). Lp1 also had a high copy-number *Pst*I fragment of 6.4 kb, not derived from the mtDNA. Blot-hybridization analysis (not shown) assigned this fragment and a co-migrating fragment from *E. typhina* E8 to the *rrn* repeats.

Evidence for a heteroploid nuclear genotype: Three single-conidiospore isolates from Lp1 were analyzed for *tub2* polymorphism by sequence analysis of PCR products, and both gene copies were present in each (C. L. SCHARDL, unpublished data). Likewise, Southern-blot hybridization identified both *pyr4* genes in each of six isolates from single conidiospores (M. A. COLLETT, unpublished data). To establish whether the two copies of each gene were characteristic of a single nuclear genotype, it was necessary to determine the number of nuclei in the conidiospores. Therefore, conidiospores were stained with giemsa and visualized (Figure 7). Of over 100

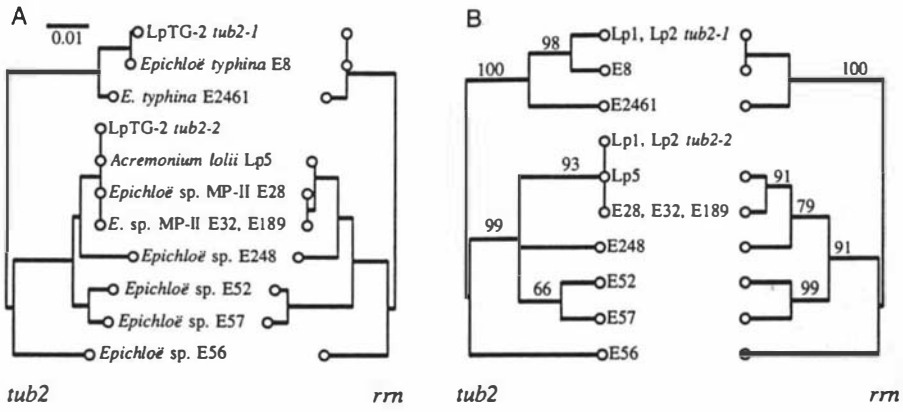

FIGURE 4.—Phylogenetic analysis of gene sequences. (A) Neighbor-joining trees on distances derived from sequences of *tub2* introns and *rrn* internal transcribed spacers. Distances were determined by the Jukes-Cantor (one parameter) method, using the aligned sequences shown in Figures 1 and 3. The bar indicates a distance of 0.01 or differences in 1% of the sites. (B) Strict consensus of the three most parsimonious cladograms derived by branch-and-bound search on the same sequences. Relative lengths of the edges are not indicated on the cladogram. Bootstrap percentages (also using branch-and-bound)are indicated for each internal edge. When informative gaps were treated as informative (see text and Figures 1 and 3) these values were slightly higher. The trees in panels A and B are unrooted, but the midpoint method on the parsimony trees (SWOFFORD 1993) placed the roots on the edges drawn at the left in the *tub2* tree, and at the right in the *rrn* tree (*i.e.*, the base of the *E. typhina* clade).

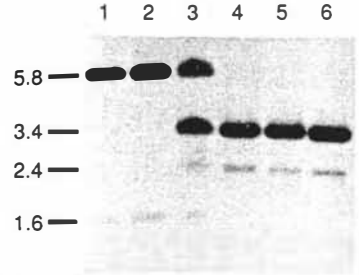

FIGURE 5.—Identification of two *pyr4* genes in endophyte LpTG-2 isolate Lp1. Sources of the DNA in each lane were: lambda clone MC12 containing the *pyr4-1* gene from isolate Lp1 (lane 1); total DNA preparations from *E. typhina* isolate E8 (lane 2); LpTG-2 isolate Lp1 (lane 3); and *A. lolii* isolates Lp5 (lane 4) and Lp9 (lane 5); and lambda clone MC11 containing the *pyr4-2* gene from Lp1 (lane 6). The DNA samples were digested with *Bam*HI, and the resulting fragments were separated by agarose gel electrophoresis, blotted, and probed with the cloned *pyr4* gene from *Claviceps purpurea*. Sizes of the hybridizing bands are indicated in kilobasepairs (kb). The *pyr4-1* pattern (5.8 kb and 1.6 kb) resembles that of *E. typhina* E8, whereas the *pyr4-2* pattern (3.4 kb and 2.4 kb) resembles that of isolates Lp5 and Lp9.

counted, all were uninucleate. Thus, the two copies of *tub2* and the two *pyr4* copies almost certainly were contained in the same nuclear genotype.

DISCUSSION

The presence in isolates Lp1 and Lp2 of two copies of nine of the ten genes investigated, but of only single copies in *A. lolii* and Epichloë isolates, indicated that endophyte taxon LpTG-2 was heteroploid. In the LpTG-2 isolates, two loci were detected for seven isozymes, and restriction fragment length polymorphisms indicated two copies each of *pyr4* and *tub2*. Molecular phylogenetic analysis of the *tub2* genes indicated that LpTG-2 was derived from an interspecific hybrid. Each *tub2* copy was linked by sequence similarity to a different clade and to a different Epichloë species. The *tub2-1* gene and *rrn* sequence indicated a particularly close relationship with an *E. typhina* isolate from perennial ryegrass. The *tub2-2* sequence was identical to those obtained from *A. lolii* and Epichloë MP-II, and could therefore distinguish neither as a likely ancestor. Allozyme profiles suggested that the other ancestor was *A. lolii*, a possibility strongly supported by the close similarity of the mtDNA profiles from *A. lolii* and LpTG-2.

A proposed scenario for the origin of the two endophyte taxa, *A. lolii* and LpTG-2, suggests two different pathways by which asexual endophytes may evolve (Figure 8). The simple scenario, whereby a pleiotropic Epichloë strain experiences a mutation that eliminates stroma expression, is omitted even though *A. lolii* could conceivably have arisen in this manner. Alternatively, a host species or genotype may suppress the stromatal stage of the fungus. For example, Figure 8 illustrates a host species transfer of MP-II as a possible origin of *A. lolii* in perennial ryegrass. Such a new host-fungus combination may not produce stromata or may do so only rarely. A different scenario, involving interspecific hybridization of co-symbiotic fungi, is proposed to account for the origin of taxon LpTG-2 (Figure 8). In general, an Epichloë spore infecting a plant that already possesses an asexual endophyte or another Epichloë strain may yield a simultaneous association of one plant with two related fungi. Because of their similar growth habits and host tissue specificity (SIEGEL *et al.* 1987), the two fungi should have ample opportunity to interact. If they are vegetatively compatible, anastomosis (hyphal fusion) may eventually be followed by karyogamy, a fusion of the dissimilar nuclei to give rise to an allodiploid hybrid. This may be followed by loss of some chromosomes or chromosome segments whose genes are redundant

TABLE 2

Allozyme profiles of *Epichloë* spp. and endophyte isolates

Species	Isolate	ACO	ALD	LAP	MDH-1	PGI	PGM-1	PGM-2
LpTG-2	Lp1	84/100	79/100	84/97	100/275	78/106	92/100	87/100
LpTG-2	Lp2	84/100	79/100	84/97	100/275	78/106	92/100	87/100
LpTG-1	Lp5	84	79	84	100	100	97	100
LpTG-1	Lp14	84	79	100	100	100	97	100
Epichloë sp. MP-II	E32	84	79	100	100	100	95	100
Epichloë sp. MP-II	E189	84	79	97	100	100	95	100
Epichloë typhina MP-I	E8	100	100	97	275	106	100	87
E. typhina MP-I	E2461	84	100	94	275	88	100	74

Numbers indicate allozyme migration relative to that of a reference strain, as in CHRISTENSEN *et al.* (1993). Enzymes are aconitase (EC no. 4.2.1.3) (ACO), aldolase (EC no. 4.1.2.13) (ALD), leucine aminopeptidase (EC no. 3.4.11.1) (LAP), malate dehydrogenase (EC no. 1.1.1.37) (MDH-1), phosphoglucose isomerase (EC no. 5.3.1.9) (PGI), phosphoglucomutase (EC no. 5.4.2.2) (PGM-1, PGM-2). Only homomeric bands are indicated.

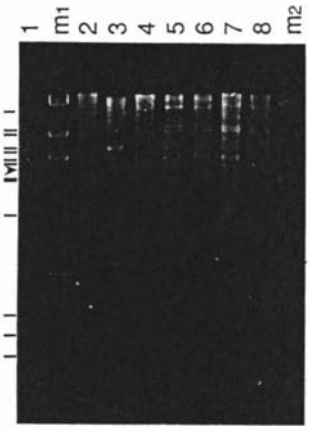

FIGURE 6.—Electrophoretograms of fungal DNA cleaved with *Pst*I. Samples are mtDNA from isolate Lp1 (lane 1), and total DNAs from *E. typhina* isolates E2466 (lane 2) and E8 (lane 3), LpTG-2 isolate Lp1 (lane 4), *A. lolii* isolates e44 (lane 5) and e46 (lane 6), and Epichloë MP-II isolates E32 (lane 7) and E189 (lane 8). Size markers are DNA from bacteriophage lambda cleaved with *Hin*dIII (lane *m1*) or *Hin*dIII and *Eco*RI (lane *m2*). Lines at left mark mtDNA fragments of Lp1. The black arrow head marks the 6.4-kb fragment from the rDNA repeats in Lp1 and E8. Hybridization to probes for *tub2* (Figure 2) and nuclear rDNA (H.-F. TSAI, unpublished data) indicated that the mtDNA in lane 1 was not significantly contaminated by nuclear DNA.

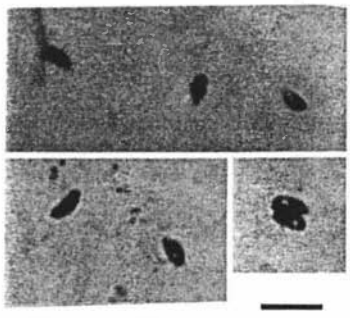

FIGURE 7.—Photomicrographs of conidiospores from LpTG-2 isolate Lp1, stained with giemsa. A single, dark staining nucleus is visible in each spore. The bar represents ten microns.

(PEBERDY 1991). For the most part, LpTG-2 appears to have retained the genes of its two ancestors, except that only one form each of ribosomal DNA and mtDNA remain.

The most obvious alternative scenario for the origin of LpTG-2 involves a rare ascospore arising from an interspecific mating of *E. typhina* and Epichloë MP-II. However, in numerous attempts at interspecific mating of Epichloë species, only barren perithecia have been obtained (C. L. SCHARDL and M. R. SIEGEL, unpublished data). Even if interspecific sexual hybrids form very rarely, they must also infect susceptible host tissue, so the likelihood of sexual hybrids producing new plant-fungus combinations is probably small. Yet, apparently, hybrid endophytes are not rare. In a survey of asexual Epichloë-type endophytes of tall fescue, no fewer than three hybridization events account for the distribution of multiple, divergent *tub2* gene copies. One endophyte taxon, FaTG-1 (= *Acremonium coenophialum*), had three *tub2* genes, each exhibiting a sequence relationship to *tub2* of a different Epichloë species. This strongly suggests that FaTG-1 originated as a hybrid of an Epichloë species with a fungal ancestor that was, itself, a hybrid. So far, no known sexual strain of Epichloë exhibits the indications of heteroploidy, such as multiband allozyme profiles (CHRISTENSEN *et al.* 1993; LEUCHTMANN and CLAY 1990) and multiple *tub2* gene copies (TSAI *et al.* 1994). Thus, the existence of a triple hybrid like FaTG-1 suggests that vegetative hybridization can occur in this group of fungi.

A potential barrier to interspecific hybridization of fungi *in planta* is vegetative incompatibility (LESLIE 1993). At present, there is little information regarding vegetative incompatibility in Epichloë species, their asexual relatives, or any other Clavicipitales. However, fungal behavior in mating tests suggests that Epichloë does not exhibit vegetative incompatibility. The first change observed when a stroma of one mating type is inoculated with spermatia of the other mating type is a thick, spreading mycelium (SCHARDL and TSAI 1992). This is probably heterokaryotic because, within several days it gives rise to raised bumps which, in intraspecific mating, mature into fertile perithecia. The initial my-

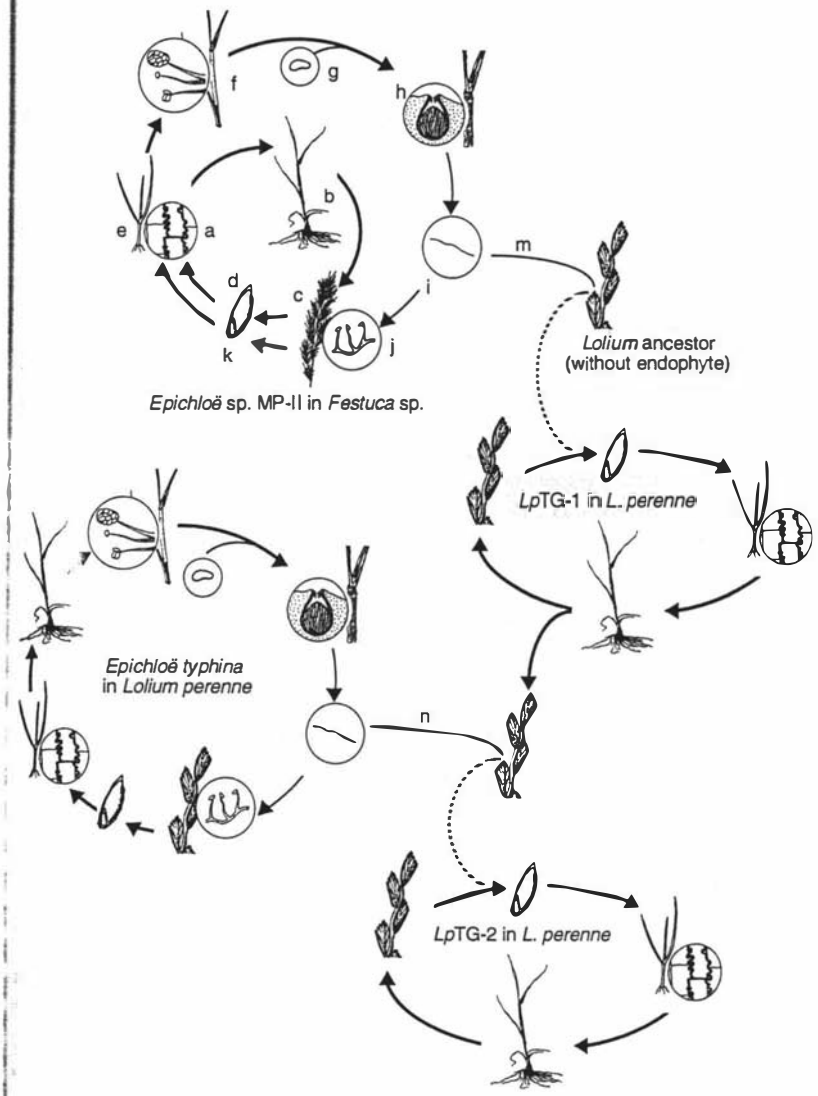

FIGURE 8.—Possible evolutionary origins of *Acremonium lolii* (=LpTG-1) and LpTG-2, endophytes symbiotic with perennial ryegrass. The postulated ancestor of *A. lolii* is Epichloë MP-II, whose alternative sexual and asexual life cycles in association with its hosts (species of genus Festuca) are depicted at top. Fungal structures are shown in circles. In the asexual cycle, highly convoluted hyphae grow intercellularly in leaf sheaths (a), floral meristems (b), and in the ovules of the florets (c) such that the fungus is transmitted in the seed (d). In the sexual cycle, the fungus also grows intercellularly and asymptomatically in vegetative leaf sheaths (e), but then produces a stroma around the immature host inflorescence arresting its development (f). Fertilization occurs by transfer of spermatia between stromata of opposite mating types (g). If the parents are conspecific (same mating population), perithecia containing asci develop (h), and filamentous ascospores are ejected (i). Germinating ascospores, initiating cycles of asexual sporulation (conidiation), are postulated (though not demonstrated) to cause infection of host florets (j) and ultimately of seed (k) (BACON and HINTON 1991). Infection by an Epichloë MP-II ascospore of a floret of a perennial ryegrass ancestor (m) may have given rise to *A. lolii* symbiosis. This symbiosis is maintained in successive plant generations by seed transmission. It is further suggested that infection of a perennial ryegrass/*A. lolii* symbiotum by *Epichloë typhina* (n), whose life cycle is depicted at the left, resulted in co-infection of the ryegrass plant, and that *A. lolii* and *E. typhina* subsequently underwent anastomosis and karyogamy to form the interspecific hybrid. Either the hybrid entity was already nonpathogenic, or subsequent genetic alterations yielded the endophyte taxon LpTG-2.

celial growth has been consistently observed in attempted matings between opposite mating types, regardless of whether the stromatal and spermatial parents are of the same mating population (SCHARDL and TSAI 1992). This observation suggests that there is no genetic system in Epichloë suppressing proliferation of heterokaryotic mycelia.

Heterokaryotic euascomycetes have been generated in the laboratory by anastomosis of vegetatively compatible hyphae or by fusion of protoplasts (PEBERDY 1991). Nuclei in heterokaryons fuse on rare occasions to produce diploids (or polyploids), which may lose chromosomes to give aneuploids and, eventually, haploids. This process of generating new genetic combinations without an intermediate meiotic stage is termed parasexuality. Whether hybridization or parasexual cycles play any significant role in nature has remained unknown either because they occur very rarely (GLASS and KULDAU 1992) or because natural hybrids have escaped notice. The genotypes of LpTG-2 and of certain endophyte isolates from *Festuca arundinacea* (TSAI *et al.* 1994) indicate such hybrids exist, at least in this group of euascomycetes. Previous studies of allozyme patterns (CHRISTENSEN *et al.* 1993; LEUCHTMANN and CLAY 1990) have also suggested that many asexual endophytes are heteroploid (or perhaps sometimes heterokaryotic) in contrast to their haploid, sexual relatives, suggesting that interspecific hybridization between Epichloë and endophytes may be common.

It is interesting to consider the consequences to the host of hybridization between *A. lolii* and *E. typhina*. The choke pathogen isolate E8, introduced into a number of *L. perenne* plants from various cultivars, has consistently exhibited the ability to sterilize these plants (M. R. SIEGEL, personal communication). If this fungus infected plants containing *A. lolii*, the choke stage of *E. typhina* would be antagonistic both to the plant and to the seed-disseminated endophyte. Sexual recombination of the host plant would be eliminated, and the plant (with endophyte and pathogen) would be restricted to localized and vegetative propagation via tillers. At this point, some genetic change in the symbiotum–either

within the plant or fungal genome–that releases the restriction on production of seed will be of obvious selective advantage to the plant. In the origin of LpTG-2, this genetic change involved hybridization of the two fungal species, and yielded either immediately or eventually a new, seed-borne and non-pathogenic endophyte. This scenario adds a twist to the concept of evolution of mutualists from antagonists (Loeschcke and Christiansen 1990; Michalakis *et al.* 1992). If the mutualists tend to lose the sexual cycle (Law and Lewis 1983), they may exhibit mechanisms for evolution and diversification very different from those of their sexual cousins. Significantly, *A. lolii* (and perhaps other grass endophytes) can apparently disarm a related antagonist by genetic hybridization.

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