CHARACTERISATION OF PUTATIVE AVIRULENCE GENES IN FLAX RUST

Melampsora lini

by

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Abstract

The aim of this study was to isolate and characterise four linked avirulence genes (A-L5, A-L6, A-L7, A-Lx) from a homozygous strain of flax rust (*Melampsora lini*). A 250 bp clone (pERT 5.8) was isolated by subtractive hybridisation between DNA of a rust strain, 228Θ9Θ, homozygous for the 4 linked avirulence genes, and DNA from a mutant, Aec68.1, used as the subtractive driver (Timmis *et al.*, 1990). The rust strain Aec68.1 is a close relative of 228Θ9Θ but has the four linked avirulence genes deleted (Timmis *et al.*, 1990). Genetic evidence suggested that pERT 5.8 was within, or linked to, these avirulence genes. Six clones were isolated from an EMBL4 genomic library using pERT 5.8 as a probe. One lambda clone was partially characterised in this project through a combination of sequencing, mapping, computer sequence analysis and PCR amplification of RNA transcripts.

Southern hybridisation of Aec68.1 and 228Θ9Θ DNA using the subcloned *Eco*RI fragments of lambda clone 4 indicated that at least 2 kbp of DNA (subcloned as pMl4N) was deleted in its entirety from the genome. The extent of the deletion in Aec68.1 may be much larger because sequences flanking pMl4N apparently contain dispersed repeats present in both strains.

Northern analysis of total RNA from both a compatible (growth of rust) and incompatible (no growth of rust) reaction between appropriate flax varieties and 228Θ9Θ using the insert from pMl4N as a probe was insufficiently sensitive to detect any mRNA produced by this region of the genome. Sequence analysis of the insert from pMl4N identified a large open reading frame of 840 bp to which primers were designed for PCR analysis of possible gene products. These primers amplified a specific transcript from total

RNA isolated from germinated 228090 rust spores alone and from total RNA isolated from 228090 infected plants. No PCR product was detected in the incompatible reaction.

Another rust strain, 271.26, virulent on hosts differentiating these four specificities (a-L5, a-L6, a-L7, a-Lx) contains DNA homologous to pMl4N and virulence in this strain is therefore not due to deletion of a segment of DNA. PCR analysis of total RNA from germinated 271.26 spores alone and from a compatible reaction between flax and this rust strain also indicated the presence of a transcription product of the 840 bp open reading frame.

Comparisons between four cDNA clones of 228090 and 271.26 mRNAs indicated that 228090 produces transcripts from at least two different genes and 271.26 produces transcripts from four different genes. This may indicate the possibility that a small gene family produces several different mRNA products.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference had been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for photocopying and loan.

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Abbreviations:

α-^{32}P-dATP - alpha labelled deoxyadenosine triphosphate

α-^{32}P-dCTP - alpha labelled deoxycytidine triphosphate

A - avirulence

BSA - bovine serum albumin

bp - base pairs

cDNA - DNA complementary to RNA

CsCl - caesium chloride

Da - daltons

°C - degrees Celsius

dH_2O - deionised water

Ci, μCi - Curies, microCuries

dATP - 2'-deoxyadenosine-5'-triphosphate

dCTP - 2'-deoxycyidine-5'-triphosphate

ddATP - 2',3'-dideoxyadenosine-5'-triphosphate

ddCTP - 2',3'-dideoxycytidine-5'-triphosphate

ddGTP - 2',3'-dideoxyguanosine-5'-triphosphate

ddNTP - 2',3'-dideoxy-nucleoside-5'-triphosphate

dGTP - 2'-deoxyguanosine-5'-triphosphate

dTTP - 2'-deoxythymidine-5'-triphosphate

DNA - deoxyribonucleic acid

DNase I - deoxyribonuclease I

DTT - dithiothreitol

EDTA - ethylene diamine tetra acetic acid

γ-^{32}P-dATP - gamma labelled deoxyadenosine triphosphate

x g - gram/force of gravity

g, mg, μg, ng - gram, milligram, microgram, nanogram

hr - hour

HR - hypersensitive response

IPTG - isopropylthioβ-galactoside

kbp - kilo basepairs

λ - bacteriophage lambda

L, ml, μl - litres, millilitres, microlitres

mA - milliAmperes

min - minute

Mr - relative molecular mass

mRNA - messenger RNA

M, mM, μM - moles, millimoles, micromoles per litre

O.D. - optical density

ORF - translational open reading frame

p - plasmid

PCR - polymerase chain reaction

% (w/v) - percentage weight per volume

% (v/v) - percentage volume per volume

R - resistance

RNA - ribonucleic acid

RNase A - ribonuclease A

sec - second

SDS - sodium dodecyl sulphate

TCA - trichloroacetic acid

Tris - Tris(hydroxymethyl)amino methane

X-gal - 5-bromo-4-chloro-3-indoyl-β-galactoside

Chapter 1
Literature review

1.1 Introduction

Although plant diseases are caused by many different pathogens which include viruses and prokaryotes with comparatively small genomes, the majority of crop damage is caused by the higher fungi which contain complex eukaryotic genomes (Timmis and Whisson, 1987). Fungal diseases are known in virtually all natural and cultivated populations of plants (Ellis *et al.*, 1988). The rusts and powdery mildews of commercially important cereal crops are a major cause of reduction in yields and thus have been the subject of intense study for more than half a century (Ellis *et al.*, 1988; Flor, 1956). Despite efforts to maintain host resistance to fungal diseases through breeding and the widespread use of fungicides, these diseases significantly reduce yields of many agricultural and horticultural plants (O'Dell *et al.*, 1989; Ellis *et al.*, 1988). This is largely because of the continual erosion of the effectiveness of host varietal resistance and of fungicides by pathogen evolution (O'Dell *et al.*, 1989).

The advantages of breeding for disease resistance have resulted in research which has contributed knowledge about the inheritance of disease resistance in plants (Ellingboe, 1976). In view of the complexity of fungal-plant relationships it is ironic that early genetic work utilised complex fungal pathogen interactions with plants (Timmis and Whisson, 1987). This knowledge of the genetics of a few systems provides a platform on which to base molecular approaches to fungal diseases of plants(Timmis and Whisson, 1987).

1.2 Plant-pathogen interactions

Microorganisms require specific genetic information to colonize plant tissues and establish symbiotic or parasitic relationships (Keen and Staskawicz, 1988). In order to colonize host tissue and reproduce, a successful parasite must have accumulated the genetic information to eliminate, overcome, avoid or escape all of the host defenses encountered (Keen, 1982a). A key objective in studying host-pathogen interactions is to determine how a pathogen evolves to overcome the defenses of a previously resistant host (Kearney *et al.*, 1988).

Plants come in contact with a variety of microorganisms during their lifetime. Some of them may not interact with the plants, some are symbiotic eg. *Rhizobium*, and others are pathogenic (Boller, 1987). Plants are known to employ many tactics to avoid and combat infection by pathogens (Rigden and Coutts, 1988). The series of events that follows the initial encounter between a potential host plant and a pathogen may involve constitutive or induced defenses by the host and aggressive or counteractive responses by the pathogen (Dunkle, 1984).

Several disease resistance mechanisms are operative in plants; some act preinfectionally, others postinfectionally (Hooker and Saxena, 1971). The preinfection defense mechanisms are in the form of preformed physical and chemical barriers (Hooker and Saxena, 1971). Failure of parasitism may occur in some host-parasite interactions during attempted penetration of cuticle and epidermal walls but the best-known defense mechanisms of plants operate after penetration into the cells (Deverall, 1977). Postinfection defense mechanisms involve the inactivation of extracellular toxins and enzymes of the pathogen, the production of morphological changes in the tissue of the host to isolate the pathogen, or the production of abnormal metabolites inhibitory or lethal to the pathogen (Deverall, 1977). Preformed chemical resistance factors exist in the host before infection, and they are toxic to the infecting agents (Goodman *et al.*, 1986). Pre-existing antimicrobial substances found in host plants include an array of phenolic compounds, phenolic

glucosides, glucose esters and benzoxazolinones (Goodman *et al.*, 1986).

In plant cells the responses to extracellular signals are many and varied. The ability of a host to suppress or retard the activity of a pathogen may depend on many factors:

Phytoalexin accumulation: Phytoalexins are generally defined as low-molecular weight antimicrobial compounds that are synthesised by and accumulate in plants after exposure to microorganisms (de Wit, 1987).

Lignification: Enhanced cell wall lignification has been observed in a number of plant species following challenge by various plant pathogenic fungi, viruses, nematodes and treatment with elicitors (Collinge and Slusarenko, 1987). Histochemical studies show that lignification often occurs before penetration, as well as during colonisation, in either the epidermis or the internal cells of many plant organs (de Wit, 1987).

Hydrolytic enzymes: Endochitinase activity increases in many plants after inoculation with fungal, bacterial and viral plant pathogens (Collinge and Slusarenko, 1987). Its substrate, chitin, does not occur commonly in higher plants but it is present in the cell walls of many fungi (Lucas *et al.*, 1985). Plant chitinases have been shown to be potent inhibitors of fungal growth (Collinge and Slusarenko, 1987). Endochitinase also shows lysozyme activity and its induction is thought to be an effective defense mechanism of plants against invading bacteria (Roberts and Selitrennikoff, 1988). β-1,3-glucanase can also act to release elicitor-active carbohydrate fractions from β-1,3-glucans of fungal cell walls (Collinge and Slusarenko, 1987). A much studied group of phytopathogenic fungi, the Oomycetes, contain cellulose rather than chitin in their cell walls (Boller, 1987). It is speculated that the cell walls of such fungi are a target for plant cellulase (Boller, 1987). Trehalase has been found in tissue cultures of several plant species (Boller, 1987). Its substrate, trehalose, has never been conclusively demonstrated in higher plants but is common in fungi and bacteria (Boller, 1987).

Proteinase inhibitors: Polypeptide inhibitors of protein hydrolysing enzymes are widely distributed in all plant tissues and are thought to have a role in defense against

herbivores since they tend to be active against animal and not endogenous proteases (Collinge and Slusarenko, 1987). Cell-wall polysaccharides from plants have been regarded as resistance factors because insect and microorganism proteinases are inhibited by cell wall fragments released after cell damage (Goodman *et al.*, 1986).

Pathogenesis-related proteins: Pathogenesis-related proteins are induced in a number of plant species following infection (Kauffmann *et al.*, 1987). They have the common properties of being acid-extractable, protease-resistant, of extracellular location and of relatively low Mr (Collinge and Slusarenko, 1987). Their roles and functions in stress and disease resistance are uncertain.

Agglutinins (lectins): These compounds are host membrane-bound or cell wall-bound proteins or glycoproteins that specifically bind to carbohydrates or to molecules containing carbohydrates on the surface of the pathogen (Goodman *et al.*, 1986).

Other proteins: It has been shown in some instances of infection that the host cell walls accumulate hydroxyproline-rich proteins that are glycosylated with arabinose and galactose (Goodman *et al.*, 1986). Also, crude protein extracts of cell walls of several plants are capable of inhibiting the pathogen's polygalacturonase (Goodman *et al.*, 1986). Various enzymes activated in diseased plants, such as peroxidases, glycosidases and ribonucleases have frequently been ascribed a role in host-pathogen compatibility or incompatibility (Goodman *et al.*, 1986).

The hypersensitive reaction (HR): Infection in many plants is isolated and further spread of the pathogen prevented because of cell death and necrosis in the region surrounding the infection (Rigden and Coutts, 1988). This, in effect results in the deletion or amputation of the infected region preventing further colonisation of healthy plant tissue (Pryor, 1987). The hypersensitive response is an active defense mechanism known to occur in response to infection by all known groups of plant pathogens - viruses, bacteria, nematodes, and fungi (Keen, 1990). The hypersensitive response is often associated with lignification to strengthen the plant cell walls surrounding the infection site, with the

synthesis of hydrolytic enzymes and with the accumulation of phytoalexins (Dong *et al.*, 1991).

When the hypersensitive response occurs, it is invariably associated with resistance (Collinge and Slusarenko, 1987). It appears to function as a last line of defense to pathogens after the earlier defense mechanisms are unsuccessful (Keen, 1982a).

1.3 Genetics of the flax - flax rust system

Some pathogens (necrotrophs) cause cell death of plant cells then grow by extracting nutrients from the dead cells (Ellis *et al.*, 1988). Other pathogens (biotrophs), such as those that cause rust and mildew, depend on living cells of the host plant for their nutrient supply (Ellis *et al.*, 1988). Since biotrophs must keep the plant cell alive, they tend to be highly specific, and not all isolates of a pathogen are able to attack all individuals of a host-plant species (Pryor, 1987).

Clearly, pathogens have evolved the ability to overcome the general resistance mechanisms of their hosts (Albersheim and Anderson-Prouty, 1975). However, during the evolutionary process, susceptible plants have responded to the pressure of invasion by parasites by developing more specific modes of resistance (Albersheim and Anderson-Prouty, 1975). The flax-flax rust system is extensively characterised and is an excellent example of a biotrophic system exhibiting a specific disease reaction mechanism.

Rust fungi are amoungst the most destructive plant pathogens in agricultural systems (Timmis and Whisson, 1987). All are obligate parasites and many have complex lifecycles which are difficult to manipulate experimentally (Fincham and Day, 1965). This is particularly true of wheat rust (*Puccinia graminis tritici*) which is heteroecious - requiring an alternate host, and wheat stripe rust (*P. striiformis*) which is microcyclic - having no known sexual phase (Timmis and Whisson, 1987). In contrast, the lifecycle of flax rust (*Melampsora lini*) takes place on a single host - flax (*Linum usitatissimum*) and this lifecycle may be manipulated in the laboratory (Timmis and Whisson, 1987). Most species of rusts

that have been adequately studied have been found to comprise numerous highly specialised parasitic races or strains which differ in their capacity to attack the different species and varieties of the host (Flor, 1956).

1.3.1 The pathogen - *Melampsora lini* (flax rust)

Melampsora lini, the fungal pathogen responsible for rust disease on flax, occurs on all continents (Lawrence, 1988). It has quite a wide host range in the genus *Linum*, being reported to occur on numerous European species of *Linum*, on several North American species, on the sole Australian species, *L. marginale*, and on the sole New Zealand species, *L. monogynum* (Lawrence, 1988).

Melampsora lini is a hemibasidiomycete, order Uredinales, with a probable chromosome number of n= 18 (Boehm, 1992). It is a so-called long-cycle rust which has five well defined stages, each with a characteristic spore form (Day, 1974). The lifecycle of the flax rust is described below.

1: Telia and teliospores

Telia are usually produced towards the end of the hosts growth period (Day, 1974). The teliospores of some rusts are not released, but remain attached to host debris and are a means of overwintering (Day, 1974). Flax rust overwinters as telia on flax stubble or straw left in the field or on bits of straw in uncleaned seed (Flor, 1956).

2: Basidiospores

In the spring, before germination, the two nuclei of the teliospore fuse (Day, 1974). A teliospore present on a dead stem then germinates to give a short promycelium in which meiosis occurs (Fincham and Day, 1965). The promycelium becomes septate and the cross walls delimit four haploid uninucleate cells (Day, 1974). Each cell produces a sterigma on

which a basidiospore or sporidium develops and is discharged (Day, 1974).

3: Pycnia and spermatia

If the sporidia that are discharged land on a compatible host they can initiate monocaryotic infections (Fincham and Day, 1965). Pycnia result from host infection and appear as flask-shaped mycelial structures submerged in the host tissue and opening by a stoma (Fincham and Day, 1965; Day, 1974). These pycnia appear within approximately eight days of infection (Fincham and Day, 1965). The pycnia produce small uninucleate spermatia (pycniospores), which are carried out through the opening to the leaf surface in a drop of liquid exudate (nectar) (Day, 1974; Lawrence, 1988). *Melampsora lini* is heterothallic and each pycnium is of + or - mating type (Fincham and Day, 1965). This simple mating-type system ensures that '+' spermatia will only fuse with '-' receptive hyphae, or '-' with '+' (Day, 1974). The spermatia are transferred from one pycnium to another, probably by small insects that are attracted to the nectar, water dripping or running down plants or by the coalescing of two infections growing in close proximity (Lawrence, 1988).

4: Aecia and aeciospores

Pycnia, when fertilised develop into aecia (Flor, 1956). The spermatium nucleus passes through the hyphae of the pycnium to an aecial rudiment nearby, which developed at the same time as the pycnium (Day, 1974). This migration is accompanied by nuclear division (Day, 1974). The rudiment, now dikaryotic, grows to form a pustule (the aecium), which produces chains of binucleate aeciospores (Day, 1974). These are discharged and may infect other susceptible flax plants (Lawrence, 1988).

5: Uredia and urediospores

The aeciospores give rise to dicaryotic infections which are orange pigmented dicaryotic urediospores that arise in patches under the host epidermis (Fincham and Day,

1965). The urediospore is the asexual repeating stage of the lifecycle and a new uredial generation may be produced about every ten days during the growing season (Flor, 1956).

Toward the end of the season teliospores are produced on the stems of the host (Fincham and Day, 1965). The thick-walled teliospores are resistant to adverse environmental conditions and enable the fungus to survive until the next growing season where the cycle is repeated (Lawrence, 1988).

1.3.2 The flax - flax rust interaction

The interaction between flax and its rust is one of the most extensively studied and genetically characterised examples of the interaction between a host and its pathogen (Timmis *et al.*, 1990). This knowledge stems largely from the extensive pioneering work of H. H. Flor. Flor systematically analysed the genetic interaction of rust and plant host by collecting and testing many flax varieties and rust races. From these collections were developed a panel of selected and derived flax varieties which were able to differentiate the spectrum of rust races (Flor 1935, 1940, 1941, 1942, 1951, 1954, 1955). What emerged from this research on the inheritance of reaction to rust in flax and pathogenicity in the rust was a consistent pattern of genetic interactions between the host and parasite (Ellingboe, 1984). Flor showed that host resistance (R) was almost invariably dominant to susceptibility (r) and avirulence (A) was generally dominant to virulence (a) in the rust.

1.3.3: Gene-for-gene hypothesis

Flor postulated that the interacting genes in host and parasite were complementary and there was a one-for-one correspondence between the genes in flax controlling host

reaction to rust and the interacting genes controlling pathogenicity in the rust (Jones, 1983). This one-for-one correspondence is now known as the 'gene-for-gene relationship' which was first proposed by Flor in 1956 and may be stated as follows:

'For each gene for avirulence in the parasite, there is a corresponding gene for resistance in the host'.

A host line with a R gene is not resistant to a pathogen unless the pathogen has the corresponding A gene for avirulence. Conversely, a pathogen strain with an A gene is not avirulent to a given host line unless the host has the corresponding R gene for resistance (Ellingboe, 1982). To date, 29 complementary pairs of interacting resistance and avirulence genes have been identified in the flax host and rust parasite (Timmis *et al.*, 1990). In flax, the resistance genes are clustered in five groups, K, L, M, N, and P (Flor, 1971), where the members of each group may behave functionally as closely linked genes eg. the M group, or as multiple alleles, eg. the L group (Flor 1941, 1951, 1955). The avirulence genes in the parasite are not correspondingly grouped. Eleven of these genes segregate independently of each other and the remaining 18 genes show varying degrees of linkage (Flor 1942, 1955, 1956, 1971). Thirteen of these are in four groups of apparently closely linked genes that behave as units during segregation (Lawrence *et al.*, 1988).

The validity of the complementary gene-for-gene relationship which was postulated from Flor's study of the genetics of flax rust disease has been strengthened by further genetical studies of host-parasite systems (Shintaku *et al.*, 1989). Gene-for-gene relationships have been demonstrated or suggested in at least 43 different plant interactions with pathogens, including viruses, bacteria, fungi, insects, and nematodes (Gabriel and Rolfe, 1990). The generality of complementary gene relationships between plants and pathogens suggests that there is some common mechanism by which one organism recognises another organism (Ellingboe, 1984). The gene-for-gene hypothesis is widely

accepted as valid in predicting the genetics of pathogen race specificity and plant disease resistance (Gabriel *et al.*, 1986).

According to genetic selection theories it is thought that in the absence of a selective value, avirulence genes may eventually be lost from the pathogen population (Day, 1974; van der Plank, 1968). But, given the existence of basic compatibility, there is likely to be a selection pressure on the plants towards resistance (de Wit, 1987). Once resistant genotypes emerge either naturally or in agriculture, selection pressure is again imposed on the parasite to respond by overcoming the resistance (de Wit, 1987). It has been suggested that this is the basis of gene-for-gene coevolution.

Frequency-dependent polymorphisms, whether stable or transient, may be a common result of interactions between parasites and hosts (Thompson and Burdon, 1992). Flor's studies of gene-for-gene interactions have had lasting effects on studies of the importance of major genes and frequency-dependent selection in coevolution even to the extent that they may have been overemphasised in plant breeding strategies (Thompson and Burdon, 1992).

1.3.4 Avirulence-resistance interaction

Little is known about the structure and regulation of plant disease resistance genes or about the molecular interaction of their products with the products of the pathogen avirulence genes (Keen, 1990). Avirulence is caused by recognition of the pathogen by the host resulting in active host defense (van Kan *et al.*, 1991).

If a particular plant cultivar is known to contain a single defined disease resistance gene that is not present in a second cultivar, any pathogen strain that elicits a resistant reaction only on this cultivar may be assumed to contain the complementary avirulence gene (Keen and Buzzell, 1991). If a host lacks a specific resistance gene, the corresponding avirulence gene in the parasite cannot be detected (Thompson and Burdon, 1992). In all gene-for-gene systems, resistance conditioned by a host resistance gene is completely

dependent upon the presence of a corresponding avirulence gene (Gabriel *et al.*, 1982).

In diploid host-parasite associations, the interactions occurring at any particular corresponding pair of loci may be complicated by the occurrence of resistance or avirulence loci in a heterozygous state (Thompson and Burdon, 1992). Five genetic combinations (Aa,RR; Aa,Rr; Aa,rr; AA,Rr and aa,Rr) are possible in addition to AA,RR. Nevertheless, because resistance is usually dominant to susceptibility and avirulence dominant to virulence, these combinations are generally phenotypically indistinguishable from other compatible or incompatible reactions (Thompson and Burdon, 1992). Thus, if the pathogen harbours a dominant avirulence gene (AA or Aa) and the plant host contains a complementary disease resistance gene (RR or Rr), there is no growth of the parasite - an incompatible reaction (Keen and Staskawicz, 1988). If either the pathogen lacks a functional avirulence gene (aa) or the host has a recessive allele at the corresponding resistance locus (rr) the host defense is not activated leading to successful colonisation - a compatible reaction (van Kan *et al.*, 1991).

1.4 Isolation of resistance and avirulence genes

The traditional focus of studies in the specificity of plant-pathogen interactions has been on the host (Judelson and Michelmore, 1989). An understanding of both avirulence and resistance is necessary to fully understand specificity and it is therefore important to isolate both avirulence genes and the resistance genes with which they interact (Judelson and Michelmore, 1989). In addition, if the genetic models (described later) of host-parasite interactions are correct, the avirulence gene product may be useful in isolating the products of the corresponding resistance gene products (Ellingboe, 1987). The isolation of the resistance gene product may then permit the cloning of the gene by several different strategies (Ellingboe, 1987).

1.4.1 Resistance genes

Although major genes for disease resistance have been studied extensively, very little is known about their structure (Hooker and Saxena, 1971). At present, antimicrobial resistance genes have not been cloned from any species. Genes for resistance to a plant disease are defined by their phenotypic effects and their genetic properties and not at all by their products (Ellis *et al.*, 1988).

Many research groups are trying to locate and clone the plant resistance genes defined by Flor's gene-for-gene relationship (Kerr, 1987). Several different approaches are possible. The simplest approach is to compare resistant and susceptible cultivars in the hope of finding a protein that is present in the former and absent in the latter (Kerr, 1987). Several approaches to this simplistic method have been attempted and, although the product of resistance genes have not yet been detected, the method has yielded clones of many resistance-related genes or disease response genes (Rigden and Coutts, 1988; Sharma *et al.*, 1992). Alternative approaches are available and are being actively pursued by many research groups. These include:

1. 'Shotgun' cloning of the required genes via transformation and selection for the acquisition of plant resistance (Gabriel and Rolfe, 1990). This approach was successful in the cloning of a bacterial avirulence gene (Staskawicz *et al.*, 1984) but the much larger size of plant genomes is a major obstacle (Pryor, 1987).
2. Genetic linkage analysis with a known molecular marker on a chromosome (Czarnecki and Lukow, 1992), such as restriction fragment length polymorphism (Sarfatti *et al.*, 1989) and 'chromosome walking' to the gene of interest (Young, 1990). In recent years, RFLP markers have become powerful tools in the construction of RFLP linkage maps for various plants. Many disease resistance loci have been mapped in relation to molecular markers (Sarfatti *et al.*, 1991; Segal *et al.*, 1991; Klein-Lankhorst *et al.*, 1991; Ritter, 1991).

A new DNA polymorphism assay was developed recently based on amplification by PCR of random DNA segments, using single primers of arbitrary nucleotide sequence

(Williams *et al.*, 1990). The amplified DNA fragments, referred to as RAPD (random amplified polymorphic DNA) markers, have been shown to be highly useful in the construction of genetic maps (Klien-Lankhorst *et al.*, 1991). Paran *et al.* (1991) have reported genetic maps for both lettuce and *Bremia lactucae* as part of their studies of disease resistance in lettuce.

3. Gene tagging techniques using mobile genetic elements (Bennetzen, 1988; Pryor, 1987). Gene tagging involves the inactivation of gene function by the insertion of a transposable DNA sequence (Ellis *et al.*, 1988). This strategy has already been used to isolate genes from a variety of eukaryotes, including, for example, *Zea mays* (Fedoroff *et al.*, 1984) and *Antirrhinum majus* (Martin *et al.*, 1985) as well as *Drosophila* (Bingham *et al.*, 1981). Until recently, however, only transposable elements indigenous to the species in question have been used successfully in gene identification. Fitzmaurice *et al.* (1992) have now developed a system of tagging vectors based upon the maize element *Ac*, capable of transposing into a variety of dicots including tobacco, *Arabidopsis*, carrot, potato, tomato and petunia.

1.4.2 Avirulence genes

Several avirulence genes have been isolated from a variety of systems.

Microbial avirulence genes:

The first microbial avirulence gene (*avrA*) was cloned by transformation and complementation from *Pseudomonas syringae* pv. *glycinea* (race 6) by Staskawicz *et al.* (1984). Since then, other avirulence genes from several *Psuedomonas syringae* pathovars have been cloned including *avrB* and *avrC* (Staskawicz *et al.*, 1987), *avrD* (Kobayashi *et al.*, 1990), *avrAspi1* (Vivian *et al.*, 1989) and *avrPph3* (Shintaku *et al.*, 1989). Recently, avirulence gene *avrPto* from *Pseudomonas syringae* pv. *tomato* has been reported to induce disease resistance in tomato cultivars containing the *Pto* resistance gene (Ronald *et al.*, 1992). Several avirulence genes from *Xanthomonas campestris* have also been cloned.

Gabriel *et al.* (1986) cloned 5 avirulence genes from *X. campestris* pv. *malvacearum* which interact specifically with individual resistance genes from congenic cotton cultivars (*avr*B$_2$, *avr*B$_3$, *avr*B$_6$, *avr*B$_N$ and *avr*B$_{In}$). Kearney *et al.* (1988) have identified and cloned a gene *avrBs*$_1$ specifically inducing a HR on pepper plants carrying the resistance gene *Bs*$_1$ from *X. campestris* pv. *vesicatoria*. Bonas *et al.* (1989) have also cloned an avirulence gene from *X. campestris* pv. *vesicatoria*, *avrBs3*, which was shown to be localised on a self-transmissible plasmid. Another gene, *avrBsP*, was found to cross hybridise with *avrBs3* but the two avirulence genes differed in their biological activity (Canteros *et al.*, 1991). Kelemu and Leach (1990) identified and cloned a dominant avirulence gene (*avr10*) from a race 2 strain of *X. campestris* pv. *oryzae* that corresponds with a dominant resistance gene (*Xa-10*) in rice cultivar Cas 209.

Although many of these genes have been subsequently characterised, neither their functions in the pathogen nor the mechanism by which they elicit the plant HR are generally understood.

Viral avirulence genes:

Dawson and collaborators performed key experiments indicating that the coat protein gene of tobacco mosaic virus (TMV) functions as an avirulence gene against certain strains of tobacco (Keen, 1990). Knorr and Dawson (1988) discovered that a point mutation in the wild type virus is responsible for conferring the ability of TMV to elicit the HR in *Nicotiana sylvestris*. The mutation changes the capsid protein gene to specify phenylalanine rather than serine at position 148 (Knorr and Dawson, 1988). More recently, the control of virulence has been mapped to five different point mutations occurring throughout the coat protein gene (Culver and Dawson, 1989). The identification of which viral molecule, RNA or protein, acts to elicit HR has provided a starting point for determining how this virus-host interaction takes place at the molecular level (Culver and Dawson, 1989). Culver and Dawson (1989) demonstrated that the capsid protein can act as an elicitor of the HR in tobacco and

indicates that its structural gene is an avirulence gene. This work is particularly significant since it establishes, for the first time, the biochemical function of an avirulence gene, both in the pathogen and in recognition by plants (Keen, 1990).

Fungal avirulence genes:

Two reports provide evidence for the isolation of avirulence gene products which are thought to be race-specific elicitors from pathogenic fungi (Lamb *et al.*, 1989). A partially purified galactose/mannose-rich glycoprotein from the alpha-race of *Colletotrichum lindemuthianum* has been shown to induce phytoalexin accumulation in a bean cultivar resistant to the alpha race, but not in a compatible cultivar (Tepper and Anderson, 1986). Race-specific elicitors of necrosis and chlorosis have been isolated from tomato leaves infected with *Cladosporium fulvum* (de Wit *et al.*, 1985). A necrosis-inducing polypeptide exhibits appropriate race-cultivar specificity on differential tomato cultivars containing the resistance gene complementary to the *C. fulvum avr9* gene (van Kan *et al.*, 1991). It has been hypothesised that the avirulence of fungal races on Cf9 genotypes is due to the production of this elicitor by the avirulence gene *avr9* (van Kan *et al.*, 1991). Proof of this has been obtained by transforming a subfragment of the genomic clone of *avr9* into virulent races of Cf9 (van den Ackerveken *et al.*, 1992). Transformants in which the construct had integrated into the genome became avirulent on Cf9 (van den Ackerveken *et al.*, 1992). This indicates that the clone was completely responsible for the change in specificity from virulence to avirulence on the appropriate tomato cultivar. *Avr9* appears to be the first fungal avirulence gene to be cloned (van den Ackerveken *et al.*, 1992).

More recently in July 1992, at the Sixth International Symposium on Molecular Plant-Microbe Interactions, Barbara Valent and Forest Chumley reported the successful cloning of two fungal avirulence genes from *Magnaporthe grisea*, the causative agent of rice blast disease (see Stacey *et al.*, 1992).

1.4.3 Models for gene-for-gene recognition

Several models have been proposed to explain the molecular basis of recognition in gene-for-gene systems, but none has yet been proven by experimental evidence. Scientists working primarily with heterologous interactions, tend to support some form of elicitor-receptor model, where R and A gene products interact, not necessarily directly, to trigger a generalised defense response (Gabriel *et al.*, 1986). Others working primarily with homologous biotrophic interactions, tend to view basic compatibility as a complex, developmental process involving many genes in both host and parasite (Gabriel and Rolfe, 1990). This complex process could be easily interrupted in any number of ways by gene-for-gene interactions; the dimer hypothesis, where the primary protein products of R and A genes interact directly and are themselves a sufficient cause of a unique incompatibility (Gabriel and Rolfe, 1990). More recently, the ion-channel defense model has been proposed that combines some of the elements of the other two models (Gabriel and Rolfe, 1990). Each model will be discussed in more detail.

1: The elicitor-receptor model

This model proposes that constitutive structural elements of the incompatible but not compatible pathogen race are recognised by specific receptor molecules in the host genotype (Keen, 1982b). The primary product of the dominant allele for avirulence (A1) interacts with the primary product of the dominant allele for resistance (R1), which results in a sequence of events culminating in resistance (de Wit, 1987). A locally induced resistance response would be initiated by a putative second messenger-like substance transferring information to the host genome (de Wit, 1987). The major hypothesis is that pathogen biotypes carrying a certain avirulence gene produce a discrete elicitor substance which is specifically recognised by a receptor present in plants carrying the matching resistance gene (Keen *et al.*, 1990).

The first comprehensive elicitor receptor model was presented by Albersheim and

Anderson-Prouty (1975) who suggested a surface carbohydrate elicitor-receptor interaction. This model provided for avirulence genes encoding glycosyl transferases that modified surface proteins to provide molecular specificity recognised by host receptors encoded by resistance genes. Resistance gene receptors triggered a generalised host defense - the hypersensitive response. Keen and Bruegger (1977) later proposed a similar model, which allowed for the possibility that elicitors might be extracellular metabolites.

Although this general model has been restated in various forms, the elements that remain common to the model are elicitors, receptors, and the triggering of common defense mechanisms essential to halt pathogen colonisation in both homologous and heterologous interactions.

2: The dimer model

This model hypothesised by Ellingboe (1982) proposed that the primary product of an A gene in the pathogen interacts with the primary product of the corresponding R gene in the host to form a dimer. The monomers are not active in bringing about an incompatible relationship but the dimer is (Ellingboe, 1982). The monomers are viewed as directly binding to one another in a protein-protein interaction which causes incompatibility. According to the dimer model, the hypersensitive response is viewed as a consequence, but not a cause, of incompatibility (Gabriel and Rolfe, 1990). This proposal would predict a strict genetic one-for-one relationship when inheritance of the interactions is examined in both host and parasite (Ellingboe, 1982). This model is supported by the observation of intermediate hypersensitive response that could be due to a weaker binding of the molecules involved (Herbers *et al.*, 1992).

3: The ion-channel defense model

This model was proposed by Gabriel *et al.* (1988). It is consistent with the elicitor-receptor model in providing for the recognition of endogenous and exogenous elicitors, and

it emphasises the ability to recognise metabolites that result from avirulence gene activity (Gabriel and Rolfe, 1990). In both the elicitor-receptor and ion-channel defense models, resistance genes were proposed to encode transmembrane protein receptors. However, the ion-channel model is closer to the dimer model in emphasising the role of specific virulence factors in conditioning basic compatibility and would provide for the direct binding of the primary protein products of avirulence genes (Gabriel and Rolfe, 1990).

According to the model, the plant cell plasmalemma is equipped with a large number of transmembrane protein receptors capable of being opened into ion channels when bound by specific or semispecific elicitors on the outside of the cell membrane (Gabriel and Rolfe, 1990). A distinctive feature of the model is that signal transduction is due to electrolyte fluxes, in particular, by movement of calcium ions (Gabriel and Rolfe, 1990). Calcium was proposed to be of special importance, since it is well known to be a central signal ion in a large number of cellular responses, including the activation of new transcription (Gabriel *et al.*, 1988). It has been observed that several types of stress, including wounding, pathogen invasion and elicitor or toxin treatment result in electrolyte leakage from affected plant cells (Gabriel *et al.*, 1988).

The molecular interactions between plant hosts and their fungal parasites are still unclear. So, to study the action of genes involved in this relationship it is necessary to isolate the genes involved.

The following study is aimed at isolating and identifying avirulence genes in flax rust. The work presented involves characterisation of a lambda clone thought to contain genomic DNA of one or more of four linked avirulence genes (A-L5, A-L6, A-L7, A-Lx) found in flax rust. Characterisation of this lambda clone includes a combination of sequencing, mapping, computer sequence analysis and transcription studies.

Chapter 2
Materials and methods

2.1 Materials

General reagents

General chemical reagents were of analytical research grade and were purchased from a variety of manufacturers. Ampicillin, chloramphenicol, IPTG, X-gal and deoxyribonucleotides were purchased from Boehringer-Mannheim. Sepharose CL-4B was supplied by Sigma Chemical Company. Sepharose CL-6B was supplied by Pharmacia.

Enzymes

Restriction endonucleases (and 10X reaction buffers), proteinase K, pronase, phosphatase (alkaline), RNase free DNase I and Klenow were purchased from Boehringer Mannheim. RNase A, lysozyme and deoxyribonucleic acid (type III from salmon testes) were purchased from Sigma Chemical Co. Mung-bean nuclease and exonuclease III were purchased from Pharmacia. Bacteriophage T4 DNA ligase was purchased from New England Biolabs, T4 polynucleotide kinase was purchased from Bresatec Pty. Ltd., and *Taq*1 DNA polymerase was purchased from Promega.

Radioactive isotopes

Alpha labelled ^{32}P deoxycytidine 5'-triphosphate and alpha labelled ^{32}P deoxyadenosine 5'-triphosphate (3,000 Ci/mmole) were purchased from Bresatec Pty Ltd. Gamma labelled ^{32}P adenosine 5'-triphosphate (4,000 Ci/mmole) was also purchased from Bresatec Pty Ltd.

Kits

LambdaMap System - Promega.

GeneAmp Thermostable r*Tth* Reverse Transcriptase RNA PCR Kit - Perkin Elmer Cetus.

GIGAprime DNA Labelling Kit - Bresatec Pty Ltd.

Sequenase T7 DNA Polymerase Version 2.0 Kit - United States Biochemical Corporation.

2.2 Bacterial growth media

Luria broth: 1% (w/v) NaCl, 1% (w/v) tryptone, 0.5% (w/v) yeast extract, pH 7.2

Luria agar/agarose: 1% (w/v) agar or agarose in luria broth.

Ampicillin plates: luria agar plates with 50 µg/ml of ampicillin.

LAIX plates: luria agar plates containing 50 µg/ml ampicillin, 12 µg/ml IPTG, and 40 µg/ml X-gal.

Top agar/agarose: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 0.25% (w/v) $MgSO_4$, 1% (w/v) agar or agarose, pH 7.2.

H-Top: 1% (w/v) tryptone, 0.8% (w/v) NaCl, 0.8% (w/v) agar.

2 X YT broth: 1% (w/v) yeast extract, 1.6% (w/v) tryptone, 0.5% (w/v) NaCl, pH 7.0.

M9 glucose minimal medium: 50% 2 X M9 salts, 10 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM thiamine-HCl, 0.2% (w/v) D-glucose, 3% (w/v) agar.

2.3 Stock solutions

2 X M9 salts: (per litre) 12 g Na_2HPO_4, 6 g KH_2PO_4, 1 g NaCl, 2 g NH_4Cl.

1 X Denhardt's reagent: 0.02% (w/v) ficoll, 0.02% (w/v) polyvinyl pyrrolidine, 0.02% (w/v) BSA.

1 X load buffer for agarose gels: 0.0042% (w/v) bromophenol blue, 6.67% (w/v) sucrose.

1 X load buffer for denaturing polyacrylamide gels: 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol.

SM buffer: 0.1 M NaCl, 50 mM Tris-HCl pH 7.5, 8 mM $MgSO_4$, 0.1% gelatin.

Phenol/chloroform/isoamyl alcohol: 50% (w/v) phenol extracted with 1 M Tris-HCl pH 8.0,

2.0% (v/v) β-mercaptoethanol and 0.1% (v/v) hydroxyquinoline, 48% (v/v) chloroform, 2.0% (v/v) isoamyl alcohol.

1 X SSC: 0.15 M NaCl, 0.15 M sodium citrate, pH 7.2.

1 X SSPE: 0.1 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4.

1 X TAE: 40 mM Tris-HCl, 20 mM $NaCH_3COO$, 2 mM EDTA, pH 7.8.

1 X TBE: 100 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.3.

1 X TE: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

2.4 1 X reaction buffers

Restriction endonuclease buffers were supplied with the enzymes when purchased from the manufacturer.

1 X ligation buffer: 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 0.6 mM ATP.

1 X kinase buffer: 50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine.

1 X CIP buffer: 1 mM $ZnCl_2$, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3.

1 X mung-bean nuclease buffer: 30 mM $NaCH_3COO$ pH 4.5, 50 mM NaCl, 1 mM $ZnCl_2$, 5% (v/v) glycerol.

1 X exonuclease III buffer: 50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 1 mM DTT.

1 X HIN (c-tests): 10 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 50 mM NaCl.

1 X TM buffer (DNA sequencing): 10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$

2.5 *E. coli* and lambda strains

Strains of *E. coli* and lambda that were used are presented in table 2.1.

2.6 Flax varieties and rust strains

The varieties of flax (*Linum usitatissimum*) and flax rust (*Melampsora lini*) used or referred to in this study are presented in table 2.2.

2.7 Methods

2.7.1 Flax/flax rust propagation and collection

(a) Growing the plants

Host plants were grown in steam-sterilised soil in plastic pots. Plants intended for urediospore inoculation were grown in 1 litre pots, normally with six plants per pot. The complete set of differential varieties (29 plants) plus a universally susceptible host were grown in three 3 litre pots, the varieties in each pot being planted in two concentric rings. The plants were placed in a section of glasshouse where rust was not propogated and grown at a temperature range from 14-18°C during the night to 22-24°C during the day.

(b) Inoculation and incubation

Flax plants were inoculated when they were approximately 4-5 weeks old. The plants were inoculated with urediospores by spreading about 50 mg of spores onto the surface of a few millilitres of water in a watch-glass and applying these to the leaves of the plants with a small camel-hair brush (Lawrence, 1988). The inoculated plants were then lightly sprayed with a fine mist of water and kept at high humidity overnight at a temperature below 24°C by placing them in 45 litre plastic bins with lids on (Lawrence, 1988). The plants were then removed to a separate glasshouse for the remainder of the incubation period. Just prior to the rust mycelium rupturing the epidermis and releasing urediospores the host plants were completely enclosed within a wire frame covered with colourless cellophane of the kind that is permeable to water vapour.

(c) Collection of rust

Uredospore collections were made in a room isolated from the area in which the infected plants were grown. Uredospores were collected by removing the cover and tapping plants held over a sheet of glossy paper. Short term storage of urediospores was in cotton wool stoppered glass vials at 4°C. Long term storage was achieved by placing spores into 3 ml freeze-drying ampules and evacuating for at least 30 min on a vacuum pump. The glass

ampules were heat sealed under vacuum then stored at a temperature of 2-4°C. All strains of rust were propogated from single pustules and/or tested on full sets of differential plants for contamination before use.

2.7.2 Isolation of DNA/RNA

Small-scale isolation of plasmid DNA

a. Alkaline-lysis method

Small amounts of plasmid DNA were extracted from 10 ml overnight *E. coli* cultures carrying the plasmid of interest by the alkaline-lysis method of Sambrook *et al.*, 1989.

b. LiCl method

Plasmid DNA was extracted using a modified version of the method by He *et al.* (1990). *E. coli* cells (1.5 ml) carrying the plasmid of interest were harvested from a 10 ml overnight culture by centrifugation at 12,000 x g for 1 min then resuspended in 150 µl of 2.5 M LiCl, 50 mM Tris-HCl pH 8.0, 4% (v/v) Triton X-100, 62.5 mM EDTA, followed by a phenol/chloroform extraction. The mixture was vortexed for 15 sec then centrifuged at 12,000 x g for 5 min. The upper, aqueous phase was collected and phenol/chloroform extracted again. The aqueous phase was then mixed with 1/10th volume 3 M sodium acetate pH 4.8 and 2.5 volumes absolute ethanol and the precipitated nucleic acids were harvested by centrifugation at 12,000 x g for 10 min. The pellet was then washed with 70% ethanol and recentrifuged for 5 min. After drying under vacuum the DNA was dissolved in 20 µl of 1 X TE.

Large scale plasmid DNA isolation

E. coli cells containing plasmid were grown in 500 ml Luria broth containing the appropriate antibiotic at 37°C until an $O.D._{600}$ of 0.9 was achieved. Chloramphenicol (150 µg/ml) was then added to the culture and incubated for a further 14-16 hr. Bacterial cells were harvested by centrifugation for 10 min at 10,000 x g then resuspended in 10 ml of 50

mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0, 2 mg/ml lysozyme. After incubating on ice for 30 min, 20 ml of 0.2 M NaOH, 1% (w/v) SDS was mixed into the suspended cells and further incubated on ice for 5 min. Fifteen ml of 3 M sodium acetate pH 4.8 was added and the suspension left on ice for another 60 min. The bacterial lysate was centrifuged at 25,000 x g for 15 min and the nucleic acids remaining in the supernatant were ethanol precipitated then resuspended in 1 X TE. Plasmid DNA was then purified using a caesium chloride/ethidium bromide gradient (Sambrook *et al.*, 1989).

Lambda bacteriophage DNA isolation

Lambda DNA was prepared by a modification of the protocol of Meese *et al.* (1990). Lambda bacteriophage were plated with an appropriate *E. coli* host onto agarose plates such that confluent lysis was achieved. SM buffer (3 ml) was washed across the surface of the plate for 5 hr at room temperature then the lysate mixture collected. The plate lysate mixture was spun at 1,850 x g for 5 min to remove any solid debris.

DEAE-Cellulose (DE52 Whatman) was prepared according to Meese *et al.*, 1990. The resin columns were prepared in Poly-Prep chromatography columns (Biorad). Approximately 10 ml of plate lysate was passed through 2 ml packed columns and the eluent collected. The columns were then washed with 2 ml of Luria broth and the runthrough combined with the eluate. NaCl was added to the eluate to a final concentration of 0.07 M and the phages precipitated by 2 volumes of absolute ethanol. Following centrifugation at 25,000 x g for 15 min the pellet was washed with 70% ethanol. After drying under vacuum the phage were resuspended in 1 X TE containing 0.2% (w/v) SDS and lysed by the addition of an equal volume of phenol/chloroform/isoamyl alcohol. After vortexing and collection of the aqueous phase by centrifugation the sample was phenol/chloroform reextracted and the aqueous layer collected.

The aqueous layer was then passed through a 2 ml packed sepharose CL-4B column

(equilibrated with 1 X TE) and the eluate recovered. The column was then washed through with 2 ml of 1 X TE and the runthrough combined with the eluate. The eluate was precipitated with 2 volumes of absolute ethanol, washed with 70% ethanol and after drying under vacuum, lambda DNA was resuspended in 1 X TE.

Isolation of genomic DNA/RNA

(a) Isolation of flax DNA/RNA

Leaves and stems of 4 week old flax plants (infected or uninfected) were ground to a powder under liquid nitrogen.

(b) Isolation of rust DNA/RNA

Flax rust was germinated on an air-water interface for 5 hr at 25°C. The germinated spores were then collected by filtration through Whatman 'qualitative 4' filter paper. The germinated spores were then ground to a powder in the presence of glass under liquid nitrogen.

A solution of 4% sarkosyl, 100 mM Tris-HCl pH 8.0 was added the the ground material (flax or rust) and allowed to thaw. The mixture was spun at 1,850 x g for 5 min and the supernatant collected. This step was repeated before measuring the volume of the supernatant. To this, 1 g/ml (w/v) of CsCl was added. Approximately 10 ml of this solution was added to a 13.5 ml ultracentrifuge tube (Beckman) and underlayed with approximately 3.5 ml of 0.965 g/ml (w/v) CsCl in 100 mM Tris-HCl pH 8.0. These tubes were spun in a Ti70.1 rotor at 100,000 x g for 16 hr at 4°C in a Beckman ultracentrifuge.

Firstly, DNA was extracted from the CsCl interface by inserting a needle (19G) into the side of the tube below the band of DNA and extracting the DNA with a syringe. The DNA was precipitated with 1/10th volume 3 M sodium acetate pH 4.8, 2.5 volumes dH_2O, and 7.5 volumes absolute ethanol. After centrifugation at 25,000 x g the pellets were washed in 70% ethanol before drying under vacuum and resuspending in 1 X TE.

The top of the tubes were then removed and the contents poured off leaving the RNA pellet. The RNA pellet in each tube was resuspended in 1 ml of dH_2O and allowed to dissolve for 30 min on ice. The suspension was spun at 1,850 x g for 1 min to pellet any undissolved debris and the supernatant collected. The supernatant was made to 0.5 M sodium acetate pH 5.2 and 5 mM magnesium acetate then precipitated with 2.5 volumes of absolute ethanol. Following incubation at -80°C for 1 hr precipitated RNA was spun at 25,000 x g for 10 min then the pellets washed in 70% ethanol, 10 mM magnesium acetate. The pellets were resuspended in a minimum volume of dH_2O after drying under vacuum.

2.7.3 Electrophoresis

DNA agarose electrophoresis

Agarose gel electrophoresis of DNA was conducted in submarine gel tanks using 1 X TAE as the running buffer. Gels were made of 1 X TAE and an appropriate concentration of agarose. DNA samples were loaded in 0.0012% (w/v) bromophenol blue, 6.7% (w/v) sucrose. Bacteriophage lambda DNA restricted with *Hin*dIII (Boehringer Mannheim) was used as a molecular size marker unless otherwise stated. DNA was recovered from agarose gels by the freeze squeeze method of Thuring *et al.* (1975).

RNA denaturing agarose electrophoresis

RNA was electrophoresed in gels consisting of 1.5% (w/v) agarose, 10 mM sodium orthophosphate pH 7.0, 8% formaldehyde with 10 mM sodium orthophosphate pH 7.0 used as the running buffer. RNA samples were denatured prior to electrophoresis by heating at 65°C for 10 min in 50% (v/v) formamide, 12% (v/v) formaldehyde, 10mM sodium orthophosphate pH 7.0.

Denaturing polyacrylamide gel electrophoresis

DNA sequencing reactions were electrophoresed at 50 mA in a gel consisting of 4% or

5% acrylamide, 7 M urea, 1 X TBE using a BRL Sequencing Gel Electrophoresis System, Model S2.

2.7.4 Controlled nested deletions

(a) Generation of nested deletions

Clone 4N was subcloned into the *Eco*RI site of the plasmid vector pUBS using standard cloning techniques (subclone designated pMI4N). Nested deletions were generated in both orientations of the subclone. For generating deletions in orientation A, 10 µg of pMI4N DNA was digested with *Hin*dIII and *Apa*I restriction enzymes. For generating deletions in orientation B, 10 µg of pMI4N DNA was digested with *Sma*I and *Bst*XI restriction enzymes. Samples of DNA were phenol/chloroform extracted and spun through a sepharose CL-6B column after each digest. The volume was then adjusted to 110 µl with 1 X TE and half removed and stored at -20°C for repeating if necessary.

To each 55 µl sample of DNA 6 µl of 10 X exonuclease III buffer was added and incubated at 37°C. After 2 min, 100 units of exonuclease III were added and mixed. Immediately, a 10 µl sample was removed from each DNA sample and added to 10 µl of 10 mM Tris-HCl pH 8.0, 10 mM EDTA then left on ice. Samples were continuously removed at 1 min intervals and placed on ice. When all samples had been removed the sample tubes were heated at 65°C for 10 min to inactivate the enzyme then returned to ice. One hundred µl of 1 X mung bean nuclease buffer containing 100 units/ml mung bean nuclease was added to each sample then incubated at 37°C for 30 min. All samples were then phenol/chloroform extracted and the aqueous layers precipitated with 1/10th volume 3 M sodium acetate and 2.5 volumes absolute ethanol. Precipitated DNA was centrifuged at 25,000 x g for 15 min and the pellets washed with 70% ethanol before vacuum dessicating and resuspending in 40 µl 1 X TE.

Twenty µl of each sample was digested with *Eco*RI and the DNA run on an 0.8% agarose gel for examination. Four µl of DNA from each appropriate timed deletions were

religated in separate ligation reactions of total volumes of 30 µl. Five µl of the ligated DNA from each sample was transformed into JM101 competent cells and plated onto LAIX colour selection plates.

(b) Screening nested deletions

Recombinant transformants were inoculated into 5 ml of Luria broth + ampicillin (50 µg/ml) and grown overnight at 37°C. LiCl small scale plasmid DNA preparations were performed on all cultures. Each DNA preparation was digested with *Pvu*II and examined by 0.8% agarose gel electrophoresis. Deletions in both orientation A and B of varying sizes were selected and DNA for sequencing was prepared.

2.7.5 Nucleic acid membrane transfer and hybridisation conditions

Southern blotting

Following electrophoresis, agarose gels were denatured in 1.5 M NaCl, 0.5 M NaOH for 30 min then neutralised in 1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 0.001 M EDTA for 30 min. DNA was then transferred to Hybond-N+ membrane (Amersham) by the method of Southern *et al.* (1975). The membrane was then alkaline fixed according to the Amersham protocol.

Southern hybridisation conditions

Membranes were hybridised with α-^{32}P-dATP labelled DNA probes in a hybridisation solution of 5 X SSPE, 5 X Denhardt's reagent, 0.5% (w/v) SDS, and 100 µg/ml of denatured, sonicated salmon testes DNA. Hybridisation was performed at 65°C for 14-16 hr. Membranes were then washed by incubating in 2 X SSC, 0.1% (w/v) SDS at room temperature for 20 min, followed by a wash at 65°C in the same solution for a further 20 min. If necessary, the solution was replaced with 1 X SSC, 0.1% (w/v) SDS and incubated at 65°C for 15 min. Autoradiography was carried out at -80°C in the presence of intensifing screens.

Northern blotting

Denaturing formamide agarose gels were soaked for 2 hr in a large volume of H_2O after electrophoresis of RNA samples. The RNA was then blotted overnight onto Hybond-C extra Membrane (Amersham) using 20 X SSC as a transfer buffer. The membrane was then baked at 80°C *in vacuo* for 2 hr.

Northern Hybridisation Conditions

Northern blot membranes were hybridised with α-^{32}P-dATP labelled DNA probes in a hybridisation solution of 50% formamide, 2 X Denhardt's reagent, 0.1% (w/v) SDS, 5 X SSPE and 100 μg/ml of denatured, sonicated salmon testes DNA. Hybridisation was performed at 42°C for 14-16 hr. Membranes were then washed by incubation in 2 X SSC, 0.1% (w/v) SDS at room temperature for 20 min. If necessary, membranes were then sequentially washed for 20 min in 0.5 X SSC, 0.1% (w/v) SDS and 0.1X SSC, 0.1% (w/v) SDS at room temperature. Autoradiography was carried out at -80°C in the presence of intensifying screens.

2.7.6 Radioactive oligo-labelling of DNA

Routinely 50-100 ng of DNA was labelled using a Bresatec GIGAprime Oligo-Labelling Kit with 25 μCi of alpha labelled ^{32}P deoxyadenosine 5'-triphosphate (Bresatec). Unincorporated nucleotides were separated from labelled DNA by passage through a column of Bio-Gel P-60 polyacrylamide beads (Bio-Rad).

DNA probes for southern hybridisation were denatured by boiling for 5 min prior to addition to the hybridisation solution. DNA probes for northern hybridisation were denatured in 0.2 M NaOH for 10 min at room temperature and neutralised with 0.2 M HCl just prior to addition to the hybridisation solution.

2.7.7 Radioactive end-labelling of DNA

Fifty ng (12.6 pmoles) of 12mer oligonucleotide DNA was labelled with 50 µCi of gamma labelled ^{32}P deoxyadenosine 5'-triphosphate (Bresatec) in the presence of kinase buffer and 15 units of polynucleotide kinase for one hr at 37°C. The enzyme was then inactivated at 90°C for 1 min.

The specific activity of the kinased oligonucleotide was determined by spotting a small aliquot onto two separate nitrocellulose filters. One filter remained untreated. The other filter was washed 3 times in cold 5% TCA for 5 min, 3 times in 70% ethanol for 5 min then allowed to dry. The amount of radioactivity associated with each filter was then determined using a scintillation counter (Beckman) and the specific activity of the γ-^{32}P-dATP labelled oligonucleotide calculated.

2.7.8 Restriction mapping of recombinant lambda DNA

(a) Partial digests

Lambda Clone 4 DNA was prepared according to the protocol 'Lambda Bacteriophage DNA Isolation' (methods). Partial restriction enzyme digests were obtained using the enzymes *Eco*RI, *Sac*I, and *Hin*dIII. A 150 µl mixture of 10 µg DNA, 1 X restriction enzyme buffer and 3 mM spermidine was aliquoted into 9 tubes; the first containing 30 µl and the remaining tubes 15 µl. One unit of the appropriate enzyme was added to tube 1, mixed, and 15 µl was aliquoted to tube 2. Tube 2 was mixed and 15 µl was added to tube 3. The serial dilution was continued to tube 8. Fifteen µl was removed from tube 8 and discarded. Tube 9 was used as an undigested control. Digests were incubated at 37°C for 1 hr. The enzyme in the samples was inactivated by heating to 65°C for 15 min. Half of each sample was viewed by electrophoresis through a 0.6% agarose gel.

(b) Hybridisation of γ-^{32}P-dATP labelled ON-R to partial digestion products

Mapping of partially restricted lambda clone 4 DNA was performed using 'The LambdaMap System' (Promega). Only the 12-base oligonucleotide complementary to the

right arm cos site (ON-R) of lambda DNA was used. A mixture of 0.1 μg of partially digested DNA, 0.1 M NaCl and 0.5 ng end-labelled ON-R was incubated at 75°C for 2 min, then at 45°C for 30 min.

γ-^{32}P-dATP was also hybridised to markers supplied with the LambdaMap System: 0.15 μg lambda marker DNA, 0.1 M NaCl, 0.5 ng end-labelled ON-R were also incubated at 75°C for 2 min then at 45°C for 30 min. After incubation, 2 μl of loading dye was added to each tube.

(c) Gel electrophoresis and autoradiography

Hybridisation mixtures were run on a 0.4% agarose gel in 1 X TAE buffer at 3 volts/cm for 17 hr. After electrophoresis the gel was placed on a large piece of 3MM filter paper (Whatman) in a shallow tray. A piece of Hybond-N+ membrane (Amersham) then 2 pieces of 3MM filter paper all cut to the size of the gel were positioned on the gel in that order. Two to three layers of blotting material was placed on top of this. An evenly distributed 1 kg weight was placed on top of the gel and allowed to compress for 2 hr. The gel and membrane were then wrapped in plastic and exposed to x-ray film for 4-12 hr in the presence of an intensifying screen.

2.7.9 DNA sequencing

Single-stranded bacteriophage DNA sequencing

(a) Preparation of DNA

The clone of interest was subcloned into the bacteriophage vectors M13mp18 and M13mp19 for single-stranded DNA sequencing using standard cloning technques. Complementary tests (c-tests) were carried out to determine the orientation of M13 clones by combining 5 μl of two single-stranded preparations and 8 μl of 10 X HIN buffer, boiling for 3 min and leaving at room temperature for 30 min prior to electrophoresis. Single-

stranded bacteriophage M13 DNA for sequencing was prepared according to Sambrook *et al.* (1989).

(b) Reactions

Sequencing of single-stranded DNA was done by the following procedure using Klenow: Bio-Labs -40 sequencing primer (2.5 ng) was annealed to 8 µl of template DNA in 1 X TM buffer by heating the mix to 100°C and then cooling slowly to room temperature. The annealed DNA was added to a tube containing 20 µCi of α-^{32}P-dCTP and 1 unit of Klenow. Two point five µl of this was aliquoted into four tubes containing one N° (table 2.3) solution (2.0 µl) and 2.0 µl of the corresponding ddNTP. The dideoxynucleotides ddATP, ddCTP, ddGTP and ddTTP were aliquoted from stock solutions of 0.3 mM, 0.05 mM, 0.15 mM and 0.5 mM, respectively.

The reactions were incubated at 37°C for 15 min and then 2.5 µl of chase (0.5 mM dCTP, 0.5 mM dGTP, 0.5 mM dTTP, 0.5 mM dATP and 0.1 units/µl Klenow) was added to each tube and the reactions incubated for a further 15 min at 37°C. Four µl of loading buffer (10 mg/ml bromophenol blue, 10 mg/ml xylene cyanol, 1 mM EDTA in deionised formamide) was added to stop the reactions. Samples were denatured at 90°C for 5 min and 2 µl of each tube loaded onto a sequencing gel.

Double-stranded plasmid DNA sequencing

(a) Preparation of template

Double-stranded plasmid DNA was isolated from 10 ml cultures of interest by the alkaline-lysis method of Sambrook *et al.* (1989).

(b) Sequencing reactions

DNA templates were sequenced by the dideoxy chain termination method of Sanger *et al.* (1977) using alpha labelled ^{32}P deoxyadenosine 5'-triphosphate and a Sequenase Version 2.0 Kit (United States Biochemical Corp.) which contains a modified T7 DNA polymerase.

Both single-stranded and double-stranded DNA sequencing reactions were electrophoresed on denaturing polyacrylamide gels. Gels were fixed in 20% (v/v) methanol, 10% (v/v) glacial acetic acid for 20 min, dried for 2 hr at 65°C and exposed to X-ray film overnight in the absence of an intensifying screen. DNA sequencing was done using either overlapping subclones, nested deletions or specifically synthesised oligonucleotide primers or a combination of all three. All DNA sequencing data presented has been bidirectionally sequenced unless otherwise stated. Sequence data was analysed using Staden, NIH programs and the Genetic Computer Group (GCG) Sequence Analysis Software Package Versions 6.1 and 7-UNIX (Devereux *et al.*, 1984).

2.7.10 Polymerase chain reaction

Polymerase chain reactions were carried out using a Gene Amp Thermostable r*Tth* Reverse Transcriptase RNA PCR Kit (Perkin Elmer Cetus) following the protocol therein. Total RNA (250-300 ng) was firstly reverse transcribed in 1 X reverse transcription buffer in the presence of 1 mM $MnCl_2$, 200 μM of each deoxynucleotide triphosphate, 0.75 μM primer 1 and 2.5 units of r*Tth* DNA polymerase. Reactions were overlayed with mineral oil (Sigma) before incubating at 70°C for 15 min.

A PCR master mix was added to the reverse trancription mixture to give final concentrations of 0.8 X chelating buffer, 1.5 mM $MgCl_2$ and 0.15 μM primer 2. Thermal cycling was carried out for 35 cycles under the temperature conditions recommended in the kit.

2.7.11 Oligonucleotide primer synthesis

All oligonucleotides that were designed were synthesised by the Department of Microbiology and Immunology, University of Adelaide.

Table 2.1 : *E.coli* and lambda strains

Strain	mcrA	mcrB	Use	Reference
E. coli				
NM538	-	+	Assay and propagation of lambda EMBL4	Raleigh *et al* (1988)
NM539	-	+	*Spi* selection of lambda EMBL4	Frischauff *et al* (1983)
JM101	+	?	Host for pUC19 and pUBS cloning	Messing (1979)
Lambda				
EMBL4			Construction of a 228090 flax rust genomic library	Frischauff *et al* (1983)

Table 2.2 Flax varieties (*Linum usitatissimum*) and rust strains (*Melampsora lini*)

Each plant contains a single different resistance gene giving a total of 29 plants (ie. a complete set of differential flax varieties). Rust strains show a growth (+) or no growth (-) reaction on each of the 29 differential plants. The genotype of avirulence genes in the rust corresponding to the L5, L6, L7 and Lx plant resistance genes are:

228090: A-L5, A-L6, A-L7, A-Lx / A-L5, A-L6, A-L7, A-Lx

Aec52: A-L5, A-L6, A-L7, A-Lx / A-L5, A-L6, a-L7, a-Lx

Aec68.1: a-L5, a-L6, a-L7, a-Lx / a-L5, a-L6, a-L7, a-Lx

271.26: a-L5, a-L6, a-L7, a-Lx / a-L5, a-L6, a-L7, a-Lx

585.01: ?-L5, ?-L6, a-L7, a-Lx / A-L5, A-L6, a-L7, a-Lx

Differential Plants	Resistance Gene	228090	Aec52	Aec68.1	271.26	585.01
Clay	K	+	+	+	-	+
Ottawa	L	-	-	-	-	-
L^1	L^1	-	-	-	±	-
Stewart	L^2	-	-	-	-	-
PBC	L^3	-	+	+	-	+
Kenya	L^4	-	-	-	-	-
Wilden	L^5	-	-	+	+	-
Birio	L^6	-	-	+	+	-
Barnes	L^7	-	±	+	+	+
B^{13}xTowner	L^8	-	-	-	-	-
Bison	L^9	+	+	+	+	+
B^6xBGS	L^{10}	-	±	±	-	±
L^{11}	L^{11}	-	-	-	-	-
L^x	L^x	-	-	+	+	+
Dakota	M	-	-	-	-	-
W Brown	M^1	-	-	-	+(±)	±
Ward	M^2	±	±	+	+	+
Cass	M^3	-	-	-	-	-
Victory A	$M^4(+M^1)$	-	-	-	-	-
Cortland	M^5	-	-	-	±	-
M^6	M^6	-	-	-	-	-
Bombay	N	-	-	-	-	-
Polk	N^1	-	-	-	-	-
Marshall	N^2	-	±	±	±	±
Koto	$P(+L^9)$	+	+	+	+	+
Armolinsr	P^1	-	-	-	+	-
Abyssinian	P^2	-	-	-	+	-
Leona	$P^3+(?)$	-	-	-	+	-
P^4	P^4	-	-	-	-	-

Table 2.3 : dNTP solutions for DNA sequencing

	A^{o}	T^{o}	G^{o}	C^{o}
0.5mM dATP	4.3µl	43µl	43µl	29µl
0.5mM dTTP	43µl	4.3µl	43µl	29µl
0.5mM dGTP	43µl	43µl	4.3µl	29µl
0.5mM dCTP	1µl	1µl	1µl	1µl
1 x TE	8.7µl	8.7µl	8.7µl	12µl

Chapter 3
Lambda clone 4 characterisation

3.1 History of the project

The primary aim of this project was to isolate and characterise avirulence genes from *Melampsora lini* (flax rust). Molecular genomic clones putatively comprising or flanking four tightly linked avirulence genes in *M. lini* have been isolated of which one was investigated in this project.

A mutant homozygous for a deletion involving four linked avirulence genes (A-L5, A-L6, A-L7, A-Lx) was produced by gamma irradiation of a rust strain, Aec52, which is genotypically A-L5, A-L6, A-L7, A-Lx/A-L5, A-L6, a-L7, a-Lx (Timmis *et al.*, 1990). A search for mutation was made by growing the irradiated spores on Lx plants which provided selection for mutation from A-Lx to a-Lx only. However all mutants obtained were also virulent on Barnes (L7). The combination of the A and a genes on a single chromosome in Aec52 was used as a control to indicate that mutants did not arise by mitotic recombination. Progeny tests of one mutant (585.01) showed that it was genotypically A-L5, A-L6, a-L7, a-Lx/a-L5, a-L6, a-L7, a-Lx, and, after selfing, a homozygous strain with the genotype a-L5, a-L6, a-L7, a-Lx/a-L5, a-L6, a-L7, a-Lx was produced. The resulting rust, Aec68.1, was virulent on L5, L6, L7 and Lx differential hosts but consistent with Aec52 at all other specificities. This mutation was assumed to have arisen by a genomic deletion because of the involvement of all four linked avirulence genes, because of the nature of the mutagen and because of the presence of the marker chromosome (A-L5, A-L6, a-L7, a-Lx) which ruled out mitotic recombination. This mutant is closely related to the strain 228090 except for other unlikely changes induced elsewhere in the genome by the

irradiation (Timmis *et al.*, 1990).

A 250 bp genomic sequence from the rust strain 228Φ9Φ homozygous for these four linked avirulence genes was isolated using the PERT (phenol emulsion reassociation technique) method (Kohne *et al.*, 1977; Kunkel *et al.*, 1985). This method allows cloning of the specific DNA sequences located within a deletion in the mutant genome by subtractive hybridisation between the 228Φ9Φ DNA (containing the four linked avirulence genes) and Aec68.1 DNA (with the four linked avirulence genes deleted). This clone (pERT5.8) containing the 250 bp insert hybridised to two *Eco*R1 restriction fragments of approximately 4.4 kbp and 2.0 kbp in genomic DNA of 228Φ9Φ, the strain homozygous for the four linked avirulence genes, but showed no homology to genomic DNA from the strain (Aec68.1) carrying the putative chromosomal deletion of these genes. The correlation between the genetically characterised deletion in Aec68.1 and the isolation of a sequence from within a region of chromosome missing from this strain of rust suggested that this 250 bp tract may be part of, or closely linked to, the target group of avirulence genes (Timmis *et al.*, 1990). Clone pERT5.8 was used to screen a genomic library of the rust strain 228Φ9Φ homozygous for the four linked avirulence genes. The genomic library consisted of 228Φ9Φ DNA partially digested with *Mbo*I that was ligated into EMBL4 digested with *Bam*HI. Six positive clones were isolated. Clone pERT5.8 hybridised to a 4.4 kbp *Eco*R1 restriction fragment from two of the lambda clones and to a 2.0 kbp *Eco*RI restriction fragment from the other four lambda clones. No clone contained both these hybridising *Eco*RI fragments (Timmis and Cerrin, unpublished).

The results presented in this study involve the characterisation of one of the EMBL4 clones isolated from the 228Φ9Φ genomic library, lambda clone 4 (LC4), which contains a 2.0 kbp *Eco*RI restriction fragment homologous to pERT5.8.

3.2 Subcloning of lambda clone 4

An *Eco*RI restriction digest of LC4 (figure 3.1) shows fragments of 19.9

kbp, 8.8 kbp, 5.0 kbp, 4.7 kbp, 2.0 kbp and 0.5 kbp. The 19.9 kbp and 8.8 kbp restriction fragments are the long and short arm of the vector EMBL4 (Frischauf *et al.* 1983). The other sized fragments of 5.0 kbp, 4.7 kbp and 2.0 kbp were designated 4V, 4A and 4N respectively and these were subcloned into the plasmid vector pUC19 (Timmis and Cerrin, unpublished).

The remaining *Eco*RI restriction fragments of LC4 were subcloned into pUC19 during the course of this project. The 2.0 kbp band seen in the LC4 digest was discovered to be a doublet (to be discussed later) and the second 2.0 kbp fragment was designated 4J. The 500 bp fragment had previously been hidden in RNA smears and when subcloned was designated 4B.

3.3: Mapping of lambda clone 4

The restriction enzyme map of LC4 was compiled from a combination of restriction enzyme analysis and sequence analysis. Subclone 4A (4.7 kbp) was sequenced with the use of nested deletions (John, unpublished). Analysis of the sequence at the *Eco*RI ends of the subclone showed that it had been attached to one of the EMBL4 arms. In EMBL4, the cloning sites and the stuffer fragment are in the order: *Eco*RI, *Bam*HI, *Sal*I-stuffer-*Sal*I, *Bam*HI, *Eco*RI (Frischauf *et al.*, 1983). The genomic DNA of 228090 digested with *Mbo*I (compatible ends with *Bam*HI) was ligated into the *BamH*I site of EMBL4 to make the library. Digestion of the clone with *Eco*RI will leave a small portion of the vector on the end of the subcloned rust DNA fragment. Analysis of restriction enzyme sites in this sequence found a single *Hin*dIII restriction enzyme site at 424 bp and a single *Sac*I restriction enzyme site at 2,984 bp from the *Eco*RI end that was attached to an EMBL4 arm.

Sequence analysis of subclone 4N (designated pMI4N) indicated that neither end of the subclone had been attached to an EMBL4 arm. A single *Sac*I restriction enzyme site was present at 311 bp from one *Eco*RI end of the subclone and no *Hin*dIII restriction

enzyme sites were present.

Subclone 4B was sequenced in both directions and found to have no *Sac*I or *Hin*dIII restriction enzyme sites (figure 3.2). Neither end of the subclone was attached to an EMBL4 arm.

Each end of subclone 4V was sequenced in one direction (figure 3.3). Analysis of the sequence found that one end had been attached to an EMBL4 arm and a *Sac*I restriction enzyme site was 267 bp from that end (figure 3.3). Restriction analysis of this fragment is shown in figure 3.4. Subclone 4V contained restriction enzyme sites for *Hin*dIII, *Sac*I and *Eco*RV in addition to its *Eco*RI ends. Molecular sizes of bands were calculated from their electrophoretic mobilities (Duggelby *et al.*, 1981) and a restriction map constructed. The estimated map of this 4V fragment confirmed the *Sac*I site at 267 bp. Another *Sac*I site was also present approximately 3.2 kbp from the *Eco*RI end attached to EMBL4. A single *Hin*dIII site was estimated to be approximately 3.8 kbp from that end. There were an estimated five *Eco*RV restriction enzyme sites in this 4V fragment.

A restriction enzyme map using the three enzymes *Eco*RI, *Sac*I and *Hin*dIII was attempted from the information obtained but a consistent map could not be compiled. Partial restriction enzyme analysis of LC4 was used as another means of obtaining a restriction map consistent with the fragment sizes observed from digestion with *Eco*RI, *Sac*I and *Hin*dIII.

LC4 was partially restricted with the three enzymes *Eco*RI, *Sac*I and *Hin*dIII (figure 3.5). A partially restricted sample of DNA should have every combination of linked fragments ranging from being completely cut to not cut at all. Each of the partially restricted samples of DNA was incubated with a labelled oligonucleotide complementary to the short arm of EMBL4. After the entire population of DNA is separated on an agarose gel only the partially restricted fragments of DNA that still have the short arm of EMBL4 attached are detected by autoradiography of the gel. In a complete population of partially digested DNA, fragments of increasing size ranging from the most digested to the least digested should be observed and their size increments will depend on which fragment is

attached to the next. Figure 3.6 shows *Eco*RI partially digested fragments increasing in size from approximately 8.7 kbp, 13.8 kbp, 14.3 kbp, 16.5 kbp, 18.2 kbp, 22.8 kbp, 27.4 kbp to 47.8 kbp which indicates that bands are attached to one another in the order 8.7 kbp (short arm of EMBL4), 5.1 kbp (4V), 0.5 kbp (4B), 2.2 kbp (4N), 1.7 kbp (4J), 4.6 kbp (4A) and 20.4 kbp (long arm of EMBL4). Figure 3.6 shows *Sac*I partial digestion products of 9.1 kbp, 12.5 kbp, 15.2 kbp, 19.7 kbp and 40.6 kbp. This indicates that fragments are joined to one another in the order, from the short arm of EMBL4 inclusive, 9.1 kbp, 3.4 kbp, 2.7 kbp, 5.7 kbp and 20.9 kbp. Figure 3.6 also shows *Hin*dIII partial digestion products of 4.5 kbp, 13.0 kbp, 17.7 kbp, 18.2 kbp, 19.5 kbp, 23.4 kbp and 42.2 kbp indicating that the fragments are attached to each other in the order, from the short arm of EMBL4 inclusive, 4.5 kbp, 8.5 kbp, 4.7 kbp, 0.5 kbp, 1.3 kbp, 3.9 kbp and 18.8 kbp.

Compared with the attempted restriction enzyme map of LC4 the partial *Eco*RI digest indicated the presence of an additional 2 kbp fragment. A *Sac*I restriction enzyme fragment that was approximately 4 kbp in the putative map was shown to be closer to 6 kbp from analysis of *Sac*I partial restriction enzyme digests. The *Hin*dIII partial digestion revealed three extra *Hin*dIII fragments that had not been previously identified. It was concluded that there was an additional 2 kbp *Eco*RI restriction fragment that contained three *Hin*dIII restriction enzyme sites.

The 2 kbp *Eco*RI restriction fragment from LC4 was isolated from an agarose gel and digested with *Hin*dIII. A 2 kbp restriction fragment remained intact (figure 3.7) which was the fragment designated 4N known to contain no *Hin*dIII restriction enzyme sites. Another two bands of 1 kbp and 500 bp (doublet) also appeared (figure 3.7). The 2 kbp *Eco*RI restriction fragment from LC4 was therefore concluded to be a doublet containing subcloned fragment 4N and the other fragment, 4J, which was subsequently subcloned. This accounted for two of the three *Hin*dIII restriction enzyme sites known to be present. Both ends of subclone 4J were sequenced in one direction and a *Hin*dIII restriction enzyme site was found 23 bp from one end (figure 3.8). This accounted for the third *Hin*dIII restriction

enzyme site discovered from partial digestions of clone 4.

After including the 4J restriction fragment, a restriction map of the enzymes *Eco*RI, *Sac*I and *Hin*dIII was obtained that agreed with the complete digests of LC4 with these enzymes (figure 3.9). LC4 consists of five *Eco*RI restriction fragments of 5.0 kbp, 4.7 kbp, 2.0 kbp, 2.0 kbp and 0.5 kbp and the entire insert is approximately 14.2 kbp in size.

3.4 Estimation of the size of the deletion from Aec68.1

It has already been shown that pERT5.8 used to screen the 228θ9θ genomic library is within a deletion in Aec68.1 and therefore unique to 228θ9θ (Timmis *et al.*, 1990). Hence, the amount of DNA known certainly to be deleted from Aec68.1 was only 250 bp. Using the subcloned fragments from LC4 the amount of DNA deleted from Aec68.1 and therefore unique to 228θ9θ was estimated. Both 228θ9θ and Aec68.1 genomic DNA was digested with *Eco*RI (figure 3.10) and Southern transferred and each subcloned fragment of LC4 was used to probe these filters. The 2 kbp insert from pMl4N hybridised to two restriction fragments of approximately 4.4 kbp and 2.0 kbp in the 228θ9θ genome (figure 3.11, track 2). There was no hybridisation to any restriction fragments in the Aec68.1 genome (figure 3.11, track 1) suggesting that the deletion in Aec68.1 is at least 2 kbp in size.

LC4, subclone 4B showed hybridisation to DNA in both rust strains but it also detected a unique restriction fragment present only in the 228θ9θ genome (figure 3.12). Subclones 4A, 4V and 4J showed hybridisation to many restriction fragments in both the 228θ9θ genome and the Aec68.1 genome (figure 3.12). These fragments could contain DNA unique to 228θ9θ (as seen with subclone 4B) but each must contain repetitive DNA dispersed within the genome of both strains. Eukaryotic genomes are known to contain non-coding DNA which is found within and between genes. Much of this non-coding DNA is repetitive and dispersed throughout the genome and similar sequences are likely to be spread widely within the species.

The actual size of the deletion is presumably larger than the 2.0 kbp observed. It is

likely that the smallest possible size of the deletion from Aec68.1 would be in the 15-30 kbp range. This is because both pERT5.8 and the 2.0kbp 4N fragment of LC4 are homologous to two *Eco*RI fragments (4.4 and 2.0 kbp) of 228090 and thus it is assumed that the sequence, and therefore possibly the avirulence genes, are present at least twice in the rust genome. If this is the case then the deletion from Aec68.1 must contain both copies of the DNA. However, the lambda clones that have been isolated from the 228090 genomic library only show homology to one or other sized restriction fragment. Consequently, these two restriction fragments, although part of the same deletion, must be at least 10 kbp apart because both fragments were not cloned together within any of the six lambda clones obtained.

3.5 Analysis of pMI4N

The 2 kbp 4N fragment present in 228090 and deleted from Aec68.1 was further analysed. The 4N fragment was sequenced using a variety of methods (figure 3.13). The 2 kbp fragment was firstly subcloned into bacteriophage M13 and sequenced. This resulted in approximately 400-500 bp of sequence data from the end of the fragment in each orientation (A and B). The end sequenced with the forward primer (orientation A) showed a *Sac*I restriction enzyme site 311 bp from the end of the fragment. The 4N fragment was then digested with *Sac*I and the two smaller fragments obtained subcloned into M13 cut with *Eco*RI and *Sac*I. The single-stranded DNA was sequenced to give a further 400-500 bp in orientation A. The smaller 311 bp *Sac*I-*Eco*RI fragment was sequenced using the reverse sequencing primer to give the orientation B sequence complementary to that already sequenced. There were no more restriction enzyme sites discovered through sequencing which would allow the fragment to be further subcloned and sequenced.

An alternative method was used to sequence the rest of the fragment. Nested deletions were made of the fragment in both orientations (figure 3.14). Each nested deletion of

appropriate size was sequenced using denatured double-stranded DNA. A nested deletion to cover the last 400 bases in orientation A could not be found and an oligonucleotide was therefore designed to cover this area of the clone.

3.6 4N nucleotide sequence analysis

The DNA sequence of both strands of the 2.0 kbp *Eco*RI fragment (4N) was determined and found to be 2010 nucleotides in length (figure 3.16). The sequence was computer analysed to search for any features of interest. The 2010 nucleotides had a %GC content of 45.3%. There were no direct or inverted repeat structures of any size or significance. The entire sequence was compared to nucleic acid data bases (Oct '92) to determine if any homology could be found between this sequence and genes of known function but no such similarities could be identified (Wilbur and Lipman, 1983).

A number of potential coding regions contained within this DNA fragment were revealed as identified by open reading frames (ORFs) (figure 3.15). All of the predicted translation start points begin at the first methionine (ATG) in the potential coding region. Infact, translation of many fungal genes begins at the first ATG except, for example, the *qa-4* gene, from *Neurospora crassa* which has four ATGs upstream of the translational start site (Gurr *et al.*, 1987). Each open reading frame greater than 20 amino acids was compared to protein databases to see if any similarities could be found between these ORFs and proteins of known function but no significant sequence similarities could be identified (Wilbur and Lipman, 1983). Computer analysis (Nov'92) of the amino acid sequences encoded by all the open reading frames in pMl4N could find no sequences indicative of N-terminus secretory peptides (von Heijne, 1985) or significant hydrophobicity compared to the fungal avirulence protein isolated (Wilbur and Lipman, 1983).

A number of bacterial avirulence genes isolated have been characterised - *avrA* (Napoli and Staskawicz, 1987), *avrB* and *avrC* (Tamaki *et al.*, 1988), *avrD* (Keen *et al.*, 1990), *avrBs1* (Ronald and Staskawicz, 1988) and *avrPph3* (Jenner *et al.*, 1991). All

avirulence proteins of chromosomal origin seem to lack secretory signal peptides and do not contain significant regions of hydrophobicity. The only example of a hydrophobic avirulence gene (*avrBs3*) was found to be on a self-transmissible plasmid in *Xanthomonas campestris* pv. *vesicatoria* (Bonas *et al.*, 1989; Herbers *et al.*, 1992). This avirulence gene, although hydrophobic, also does not appear to contain a signal peptide. The only reported fungal avirulence gene encodes a mature protein of 28 amino acids (3,192 Da). The peptide has been found to be produced as a precursor protein of 63 amino acids which contains a leader signal peptide sequence (van Kan *et al.*, 1991).

3.7 Characterisation of a potential avirulence gene, ORF1

The largest open reading frame in pMl4N (ORF 1) was examined in more detail. The putative protein product is quite large and hydrophilic and is similar in these respects to bacterial avirulence proteins already characterised. It is 840 bp in length coding for 280 amino acids (figure 3.16). Codon usage within the putative avirulence gene is shown in table 3.1. Many fungal genes that are highly expressed have been shown to have a more marked codon bias than genes expressed at a low level (Gurr *et al.*, 1987). The trends in what are considered to be highly expressed genes include a predominance of pyrimidines, especially C, at the third nucleotide position and where a rare purine is to be found in the third position, G is used in preference to A (Gurr *et al.*, 1987). The codon usage in ORF1 is not random and some degree of codon bias is present though it is not marked. Low codon bias has been found in the coding regions of several fungal regulatory genes (Gurr *et al.*, 1987).

3.8 The 5' region of ORF1

A consensus 'TATA' box is a common upstream motif in many eukaryotic genes

including those of fungi. Fungal genes may possess AT-rich sequences which bear a close resemblance to the 'TATA' box, such as TATATA, TATAA or TATAAAT. These AT-containing motifs can be as far upstream from the translational start point as 589 bp as seen with the *creA* gene from *Aspergillus nidulans* (Dowzer and Kelly, 1991) or 133 bp as seen with the *avr9* gene from *Cladosporium fulvum* (van der Ackerveken *et al.*, 1992). Although considerable variation exists in length of the 5' untranslated mRNA of fungal genes, most commonly this region is 30-70 bp in length (reviewed by Gurr *et al.*, 1987).

A likely promoter region of ORF1 is located 226 bp upstream from the presumed start point of translation (figure 3.16). The AT-rich sequence resembles a 'TATA' box with its sequence being TATAAT. This putative 'TATA' box is identical to that found in the promoter region of the *pcbC* gene from *Penicillium chrysogenum* (Carr *et al.*, 1986). Other possible 'TATA' boxes appear 130 bp and 195 bp upstream from the start point of translation.

The 'CAAT' motif is also present in the promoter regions of many eukaryotic genes (reviewed by Gurr et al., 1987). Multiple putative 'CAAT' box sequences are present upstream from the likely 'TATA' box. CATT sequences appear 29 bp and 135 bp upstream and CAAT sequences appear 185 bp, 221 bp and 320 bp upstream from the 'TATA' box (figure 3.16).

The role of 'TATA' and 'CAAT' boxes in the promoter regions of fungal genes remains unclear and whether or not they affect expression levels of the genes in which they occur can only be determined from mutational analyses of these promoters (Gurr *et al.*, 1987).

3.9 The 3' region of ORF1

Many higher eukaryotic genes are transcribed far beyond the site of polyadenylation of the mature mRNA. Addition of poly(A) usually occurs approximately 20 bp downstream from the consensus sequence AATAAA (Gurr *et al.*, 1987). Related core sequences occur in the 3' region of the potential avirulence gene at 90 bp, 223 bp and 428 bp downstream

from the stop codon and may be the signals for polyadenylation of the putative avirulence gene transcript (figure 3.16).

3.10 The potential avirulence protein (ORF 1)

ORF 1 is 280 amino acids in length and it has a predicted molecular weight of 31,650 Da. Figure 3.17 shows that the protein is quite hydrophilic but with highly hydrophobic regions clustered at the amino terminal end. The amino terminus consists of a small hydrophobic stretch of amino acids which could possibly anchor the protein in a membrane. Other hydrophobic areas of the folded protein may be the regions on the surface of the protein. From amino acid 165 onwards analysis does not reveal any significant hydrophobic stretches of amino acids (figure 3.17). This region is predicted to be quite flexible and very antigenic, with a loosely folded or linear structure. It is possible that the amino terminal end of the protein is anchored into the membrane with the carboxyl terminal end free to move about inside or outside the cell. The protein as it may be found in the cell is depicted in figure 3.18.

Although about 40% of the residues in the predicted protein are charged making the protein quite hydrophilic, there is a net negative charge resulting in the protein being acidic with an isoelectric point of 4.2. Infact, 25% of the residues are negatively charged aspartic acid and glutamic acid and the majority of these appear in clusters in the latter half of the protein (figure 3.16).

There are two asparagine-X-serine/threonine motifs that are potential sites for N-linked glycosylation (Marshall, 1972; Modrow and Wolf, 1986) located at positions 60 and 210 of the protein. Phosphorylation/dephosphorylation of proteins may regulate activities of proteins. Protein kinases alter the functions of their target proteins by phosphorylating specific serine, threonine and tyrosine residues (reviewed by Kemp and Pearson, 1990).

The Chou and Fasman (1978) and Garnier, Osguthorpe and Robson (1978) predictions of the secondary structure of the putative avirulence protein are shown in figure 3.19.

The putative avirulence protein sequence was compared with published avirulence protein sequences from both bacteria and fungi - *avrA* (Napoli and Staskawicz, 1987), *avrB* and *avrC* (Tamaki *et al.*, 1988), *avrD* (Kobayashi *et al.*, 1990), *avrBs1* (Ronald and Staskawicz, 1988), *avrBs3* (Bonas *et al.*, 1989), *avrPph3* (Jenner *et al.*, 1991) and *avr9* (van Kan *et al.*, 1991). This was done in an attempt to find similarities between this sequence and avirulence proteins to obtain further evidence for the isolation of an avirulence gene. No similarities were found but this is not surprising in view of the lack of similarities between the other isolated avirulence genes (Ronald *et al.*, 1992).

Table 3.1 : Codon usage in ORF1.

Amino acid	Codon	No.	Amino acid	Codon	No.	Amino acid	Codon	No.
Gly	GGG	7	Ser	AGT	5	Cys	TGT	0
	GGA	1		AGC	3		TGC	2
	GGT	6		TCG	1			
	GGC	4		TCA	4	Tyr	TAT	5
				TCT	2		TAC	5
Glu	GAG	15		TCC	2			
	GAA	14				Leu	TTG	4
			Lys	AAG	12		TTA	0
Asp	GAT	28		AAA	12		CTG	3
	GAC	13					CTA	3
			Asn	AAT	10		CTT	8
Val	GTG	3		AAC	6		CTC	3
	GTA	0						
	GTT	2	Met	ATG	6	Phe	TTT	7
	GTC	4					TTC	3
			Ile	ATA	3			
Ala	GCG	6		ATT	2	Gln	CAG	2
	GCA	3		ATC	5		CAA	1
	GCT	4						
	GCC	7	Thr	ACG	0	His	CAT	1
				ACA	3		CAC	1
Arg	AGG	1		ACT	5			
	AGA	3		ACC	2	Pro	CCG	0
	CGG	1					CCA	6
	CGA	5	Trp	TGG	2		CCT	5
	CGT	2					CCC	4
	CGC	3	End	TGA	1			
				TAG	0			
				TAA	0			

Figure 3.1 *Eco*RI restriction digest of lambda clone 4

0.8% agarose gel showing the *Eco*RI restriction digestion products of lambda clone 4. Track 1 contains 0.5 μg of wild type lambda DNA restricted with *Hin*dIII. Track 2 contains 0.5 μg of EMBL4 clone 4 restricted with *Eco*RI.

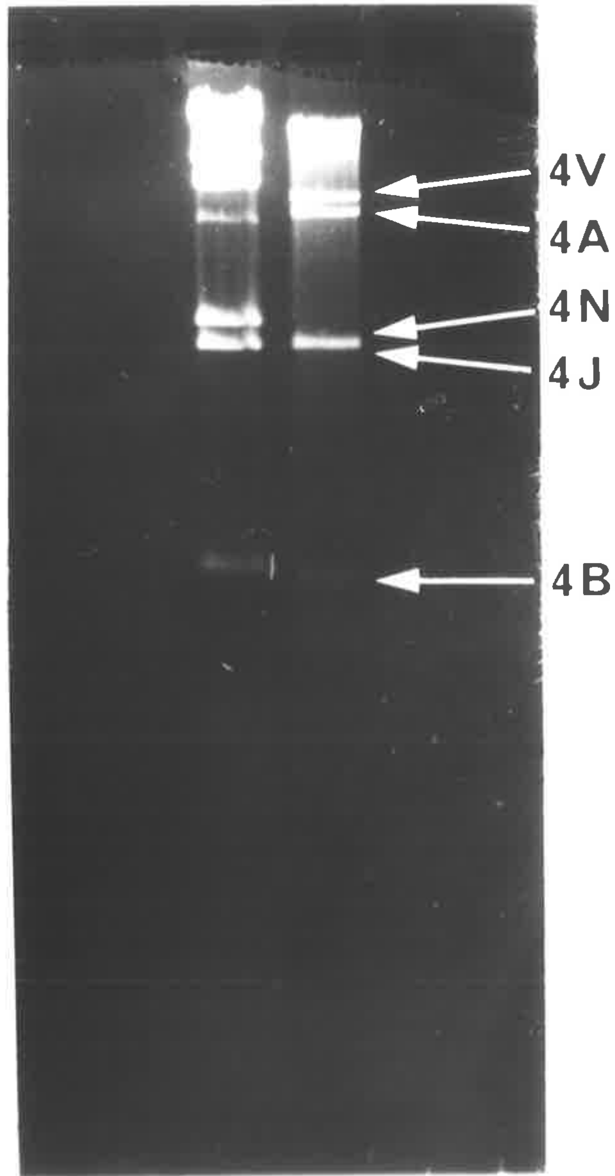

1
2
4V
4A
4N
4J
4B

Figure 3.2 DNA sequence of subclone 4B

Sequence of subclone 4B in both orientations. The 4B fragment was 522 base pairs in length and contained no *Sac*I or *Hin*dIII restriction enzyme sites.

```
      GAATTCAAGTCCGTCCCAGTGTAGATCCTTGAAGGCAATAAATAGACTGCTGTATGTCAA
  1   ---------+---------+---------+---------+---------+---------+  60
      CTTAAGTTCAGGCAGGGTCACATCTAGGAACTTCCGTTATTTATCTGACGACATACAGTT

      CCGTCTCAGTAGATTGGGAAAGCTCGTCCTTCCAGGTAGTAATATCTCGTTCGATGTAAT
 61   ---------+---------+---------+---------+---------+---------+  120
      GGCAGAGTCATCTAACCCTTTCGAGCAGGAAGGTCCATCATTATAGAGCAAGCTACATTA

      CTTTGCTTAAAAACCACCGAAGCCAGGAAGACAAAGATTGGGTAGCGTATATACTTCGCG
121   ---------+---------+---------+---------+---------+---------+  180
      GAAACGAATTTTTGGTGGCTTCGGTCCTTCTGTTTCTAACCCATCGCATATATGAAGCGC

      GAACACCGATTTGACAGGGAGGAATTTGCAGTGAAGCTGGTCTAGTCTGAGTTTTGCCTT
181   ---------+---------+---------+---------+---------+---------+  240
      CTTGTGGCTAAACTGTCCCTCCTTAAACGTCACTTCGACCAGATCAGACTCAAAACGGAA

      TATCACTGATACCTTTGAATGTCGTCTTTAAAGTGAATAAGTCTTCTTCGCAGATTCGAG
241   ---------+---------+---------+---------+---------+---------+  300
      ATAGTGACTATGGAAACTTACAGCAGAAATTTCACTTATTCAGAAGAAGCGTCTAAGCTC

      CCTTAGCAGTCTTCTTGAAAGTACAAGTCCAGGGAGCCTCAGAGATGTCATATAAGCTGA
301   ---------+---------+---------+---------+---------+---------+  360
      GGAATCGTCAGAAGAACTTTCATGTTCAGGTCCCTCGGAGTCTCTACAGTATATTCGACT

      AACAAGATCGACAGCAGATATGTTCTTTGATTTCGGGCGAGATCACTAGGCGTTTGATAA
361   ---------+---------+---------+---------+---------+---------+  420
      TTGTTCTAGCTGTCGTCTATACAAGAAACTAAAGCCCGCTCTAGTGATCCGCAAACTATT

      TTGTTCGGACATCTTTGGGTATATGGTCGCGGGTATCGTCAGCGGTAGATCCAAAGATAT
421   ---------+---------+---------+---------+---------+---------+  480
      AACAAGCCTGTAGAAACCCATATACCAGCGCCCATAGCAGTCGCCATCTAGGTTTCTATA

      CGCGGTCGCGCCTTTCTTGTTCACAAGTCAGTGCCAGAATTC
481   ---------+---------+---------+---------+--  522
      GCGCCAGCGCGGAAAGAACAAGTGTTCAGTCACGGTCTTAAG
```

Figure 3.3 DNA sequence of subclone 4V

Sequence of each end of subclone 4V in single orientations. Sequencing of subclone 4V in orientation A obtained 318 base pairs and revealed a *Sac*I restriction site 267 base pairs in from the *Eco*RI end (underlined). Sequence analysis of orientation A also revealed that this end was attached to an EMBL4 arm (underlined). Sequencing of subclone 4V in orientation B obtained 369 base pairs of DNA sequence.

Subclone 4V Orientation A

1 GAATTCCCCGGATCGCAAGGATCAATCCGAAGTTATTCCGCAAATGATGTTGCATCGGAT 60

61 GTATATGGGATTGGGTCGCAATGAAATCACCGGTCTCGTATCGGAGCCGGCATTGCATCG 120

121 GGGCGGGAGGGTGGATGGACCGTCGCTTGAAAGTTACGCGATGAGACAATTCCATCGCAC 180

181 GTAGGGTTGAGTCGAAGCAGCATGTCGCGAGGTTGCATCGGGATGAGGATCAGTCGGGTG 240

241 TCGCTCATGCGTTTTATATCGGCGCTGAGCTCCATCGGATGAGTCCGACTGTTTCTGAAT 300

301 CGGACTGGGGATTCAGTC 318

Subclone 4V Orientation B

1 GAATTCGCATAATAAAGTTCCGAGCTTTGTGACAATTGGTACGGCTGAGTCCACAGGCAA 60

61 TATGAAGCCATCCCAGAAAGATACCTACAAGGGCAACGGCTGATGGAACACCCACAAATC 120

121 AATGCTTGTGTTCAAGGAGATAGAGGGCGGTCATTCTTACCAGAAATGGAGGATGATGGA 180

181 GTCCAGTCCTCAACTGATTCGGAACCGTCCTCGTCCGACTCGTCTGATATCGAAACTTTG 240

241 TCCAGGCCTTCGCCAAGTTTTTGATGACCGACAATGAGTCGAGATCAGCTTTCTTATGTG 300

301 TGAGCTGTGTTTGACGTGACTTCCATTGGCCACGTTGATGCTGGCCCTTCTCATCGACGC 360

361 AGGTTTGGG 369

Figure 3.4 Restriction enzyme mapping of subclone 4V

Subclone 4V was individually digested with restriction enzymes *Eco*RI, *Hin*dIII, *Sac*I and *Eco*RV and with pairwise combinations of each. Sizes of the 4V restriction fragments are presented in the table. Agarose gel electrophoresis of the digestion products in columns 1-10 correspond to the columns of digestion products presented in the table above.

λ track contains 0.5 μg of wild type lambda DNA restricted with *Hin*dIII.

EcoR1	Hind111	Sac1	EcoRV	EcoR1-Hind111	EcoR1-Sac1	EcoR1-EcoRV	Sac1-Hind111	EcoRV-Hind111	Sac1-EcoRV
5.00	6.06	3.05	4.60	3.50	3.05	2.70	3.05	4.50	2.85
2.70	1.75	2.85	2.45	2.70	2.70	2.40	2.85	1.90	1.65
		2.00	0.56	1.75	2.00	1.95	1.70	0.70	1.50
			0.44		0.40	0.56	0.40	0.56	0.98
			0.15			0.40		0.40	0.56
						0.15		0.15	0.40
								0.10	0.10

Figure 3.5 Partial restriction enzyme digestion of lambda clone 4

Lambda clone 4 DNA was partially restricted with *Eco*RI, *Sac*I and *Hin*dIII. *Eco*RI partial digests are designated E1 (0.002 units/μg) and E2 (0.001 units/μg). *Sac*I partial digests are designated S1 (0.015 units/μg) and S2 (0.008 units/μg). *Hin*dIII partial digests are designated H1 (0.008 units/μg) and H2 (0.004 units/μg). 0.45 μg of DNA of the partial digests were electrophoresed through a 0.6% agarose gel. λ track contains 0.5 μg of wild type lambda DNA restricted with *Hin*dIII.

λ E1 E2 λ S1 S2 λ H1 H2

Figure 3.6 Restriction enzyme mapping of lambda clone 4

*Eco*RI (E1, E2), *Sac*I (S1, S2) and *Hin*dIII (H1, H2) partially digested DNA (0.1 μg) was hybridised with end-labelled oligonucleotide complementary to the right arm of EMBL4 (ON-R). The hybridised DNA was electrophoresed through a 0.4% agarose gel and exposed to autoradiograph film.

M - LambdaMap System markers (0.15 μg). Hybridisation with ON-R produces labelled fragments of 48.5, 38.5, 29.9, 22.6, 15.0 and 8.6 kbp (marked with arrows).

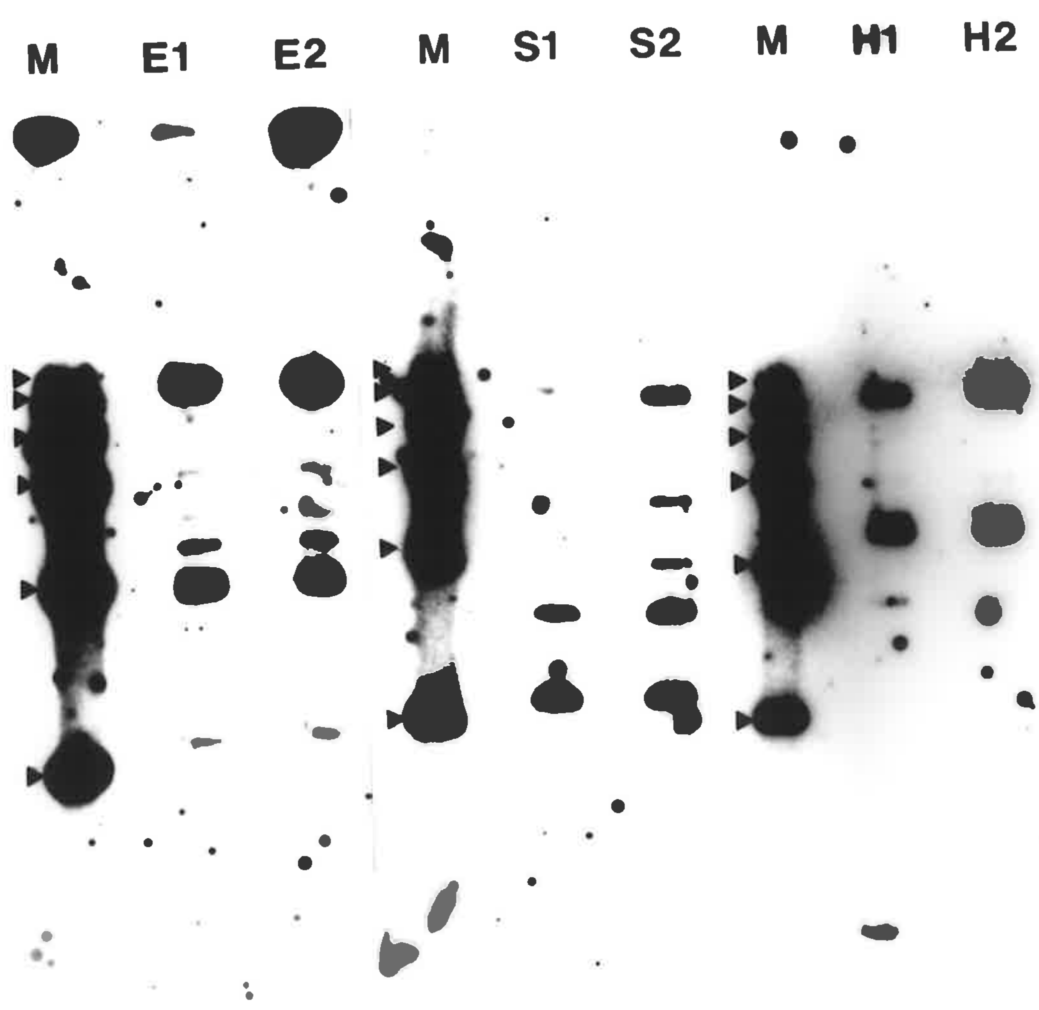

M
E1
E2
M
S1
S2
M
H1
H2

Figure 3.7 Digestion of 2 kbp fragment from lambda clone 4

The 2 kbp fragment of lambda clone 4 was digested with restriction enzyme *Hin*dIII giving bands of approximately 2 kbp, 1 kbp and 0.5 kbp (lane 2). Wild type lambda DNA (1 µg) restricted with *Hin*dIII was used as a molecular size marker (lane 1).

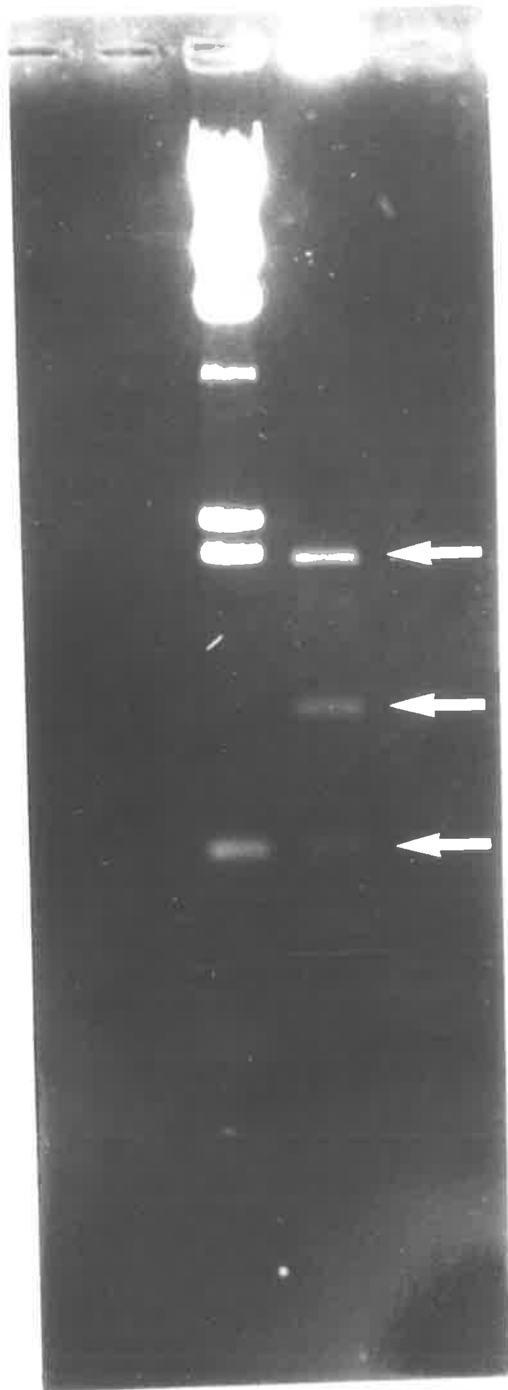

1
2
4N
4J

Figure 3.8 DNA sequence of subclone 4J

Sequence of each end of subclone 4J in single orientations. Sequencing of subclone 4J in orientation A obtained 351 base pairs of DNA sequence. Sequencing of subclone 4J in orientation B obtained 298 base pairs of sequence and revealed a *Hin*dIII restriction enzyme site 23 base pairs (underlined) in from the *Eco*RI end.

Subclone 4J Orientation A

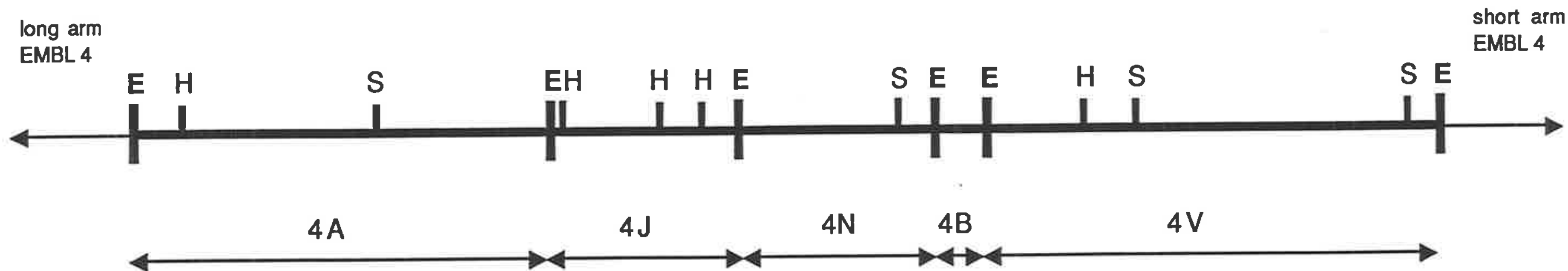
```
  1  GAATTCCTTTGGGAGCTGGCCTGGCGATAACTGTGATGGGTGACGTTTACGCTGAGGAGA   60
 61  AGGCTTTGATGAAAGGCTCGGGCGAGCACGACCGGATTTGCGTGGCACTTTGCTGGCTGG  120
121  GGTAGGAGTTGAATAGGTCTTCGAAGTCGATTGGGGCTTCAAACGGTCTCGTTTCGACGA  180
181  TGTGTCTGGAGTCGACATGGAAGTCGGCTTGGAAGTGGACTTAGAAGTGGACTTAGAAGT  240
241  GGACTTCGGGGTTGACTTGGGGGTTGACTTGGAGGTCGATTTCTTCTCGCTCTTTGATGA  300
301  GCCTCCGAGGCCCTTCATTCCTTCAATCTGACTTCAGGCCATTGATTATAG  351
```

Subclone 4J Orientation B

```
  1  GAATTCCAACTGAATGAATTACAAGCTTAAATTGAAACTGGATGAAGTATGAACTTGAAT   60
 61  TGAAACTGAATGAAGTGTAACGTGAATTGAAACTTGAGTTGAGACTAGAGAAACTGATAA  120
121  GGAATAGAGAGAAGGATGGAAGTAGCGCGCTTATGGATTCCATGTGTGTACATGATGATT  180
181  ACATGTGCGTAACCATATGTAATCTCATGTTACGCTTTGTTTTATCTTATTACTACATGT  240
241  AATTGATTGATTACATTTACTTTTGTATATGTAATAGAGGTTGTTTGTCAAGACGGAG  298
```

Figure 3.9 Restriction map of lambda clone 4

Lambda clone 4 was mapped using the restriction enzymes *Eco*RI, *Sac*I and *Hin*dIII. The long (19.9 kbp) and short (8.9 kbp) arms of the vector, EMBL4, are not to scale.



1Kb

Figure 3.10 Genomic digestion of Aec68.1 and 228090 DNA

Approximately 1 μg of genomic DNA from Aec68.1 (lane1) and 228090 (lane 2) was digested with *Eco*RI and electrophoresed through a 0.8% agarose gel. λ tracks contain 0.5 μg of wild type lambda DNA restricted with *Hin*dIII.

λ 1 2 λ

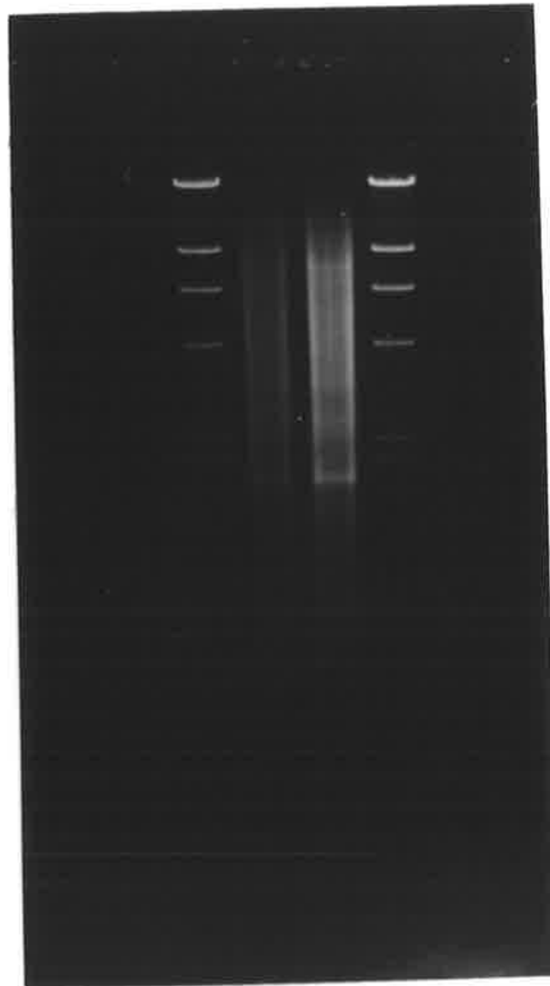


Figure 3.11 Hybridisation of the 4N fragment to genomic DNA of Aec68.1 and 228090

The 2 kbp insert of pMI4N was hybridised to a Southern blot of the agarose gel depicted in figure 3.10.

Lane 1: Aec68.1 *Eco*RI-restricted DNA

Lane 2: 228090 *Eco*RI-restricted DNA

Arrows identify restriction products of wild type lambda DNA digested with *Hin*dIII (λ).

4 N

Figure 3.12 Hybridisation of lambda clone 4 *Eco*RI restriction fragments to genomic DNA of Aec68.1 and 228090

The fragments of lambda clone 4 designated 4A, 4V, 4J and 4B were hybridised to a Southern blot of the agarose gel depicted in figure 3.10.

Lanes 1: Aec68.1 *Eco*RI-restricted DNA

Lanes 2: 228090 *Eco*RI-restricted DNA

λ tracks contain wild type lambda DNA restricted with *Hin*dIII.

4 A

λ 1 2 λ

4 J

λ 1 2 λ

4 B

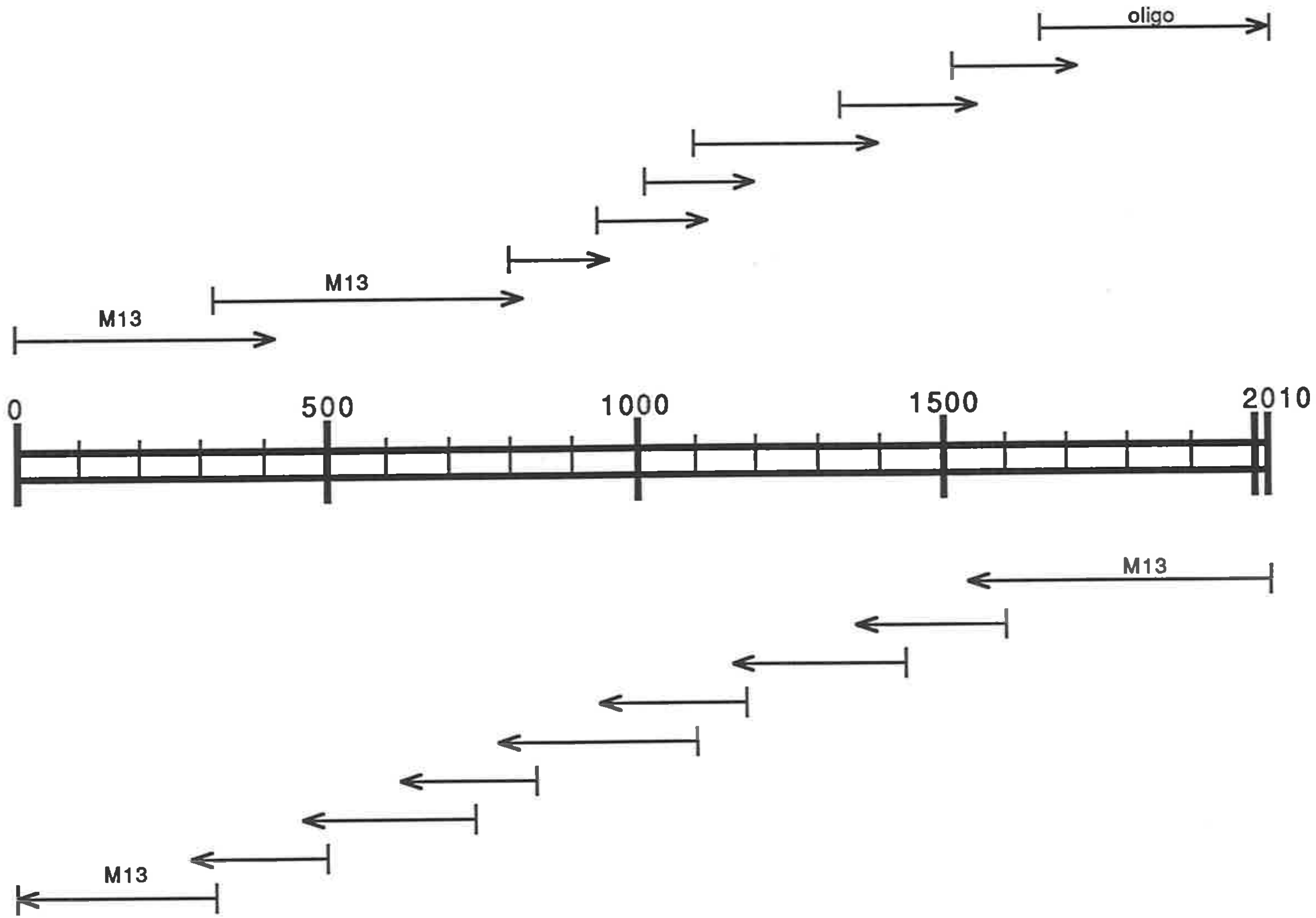
λ 1 2 λ

4 V

λ 1 2 λ

Figure 3.13 Sequencing methods of pMI4N

Sequencing of the 2 kbp 4N fragment was performed using a variety of methods. Arrows marked with 'M13' correspond to single-stranded sequencing using the M13mp18 or M13mp19 vectors. Arrows marked with 'oligo' correspond to double-stranded sequencing using a specifically designed oligonucleotide. Unmarked arrows correspond to double-stranded sequencing using nested deletions of specific sizes.



oligo
M13
M13
0
500
1000
1500
2010
M13
M13

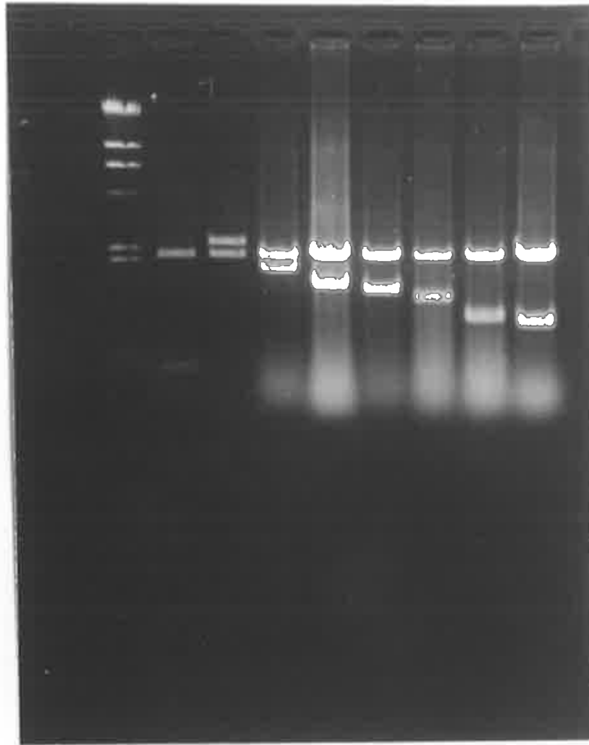
Figure 3.14 Nested deletions of pMI4N

A - subclones increasing in size of nested deletion from 1-8 in orientation A.

B - subclones increasing in size of nested deletion from 1-9 in orientation B.

λ track contains 0.5 μg of wild type lambda DNA digested with *Hin*dIII.

A λ 1 2 3 4 5 6 7 8



B λ 1 2 3 4 5 6 7 8 9

Figure 3.15 Potential translational coding regions of pMI4N

Potential open reading frames in three forward frames and three reverse frames are displayed.

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Staden Sequence

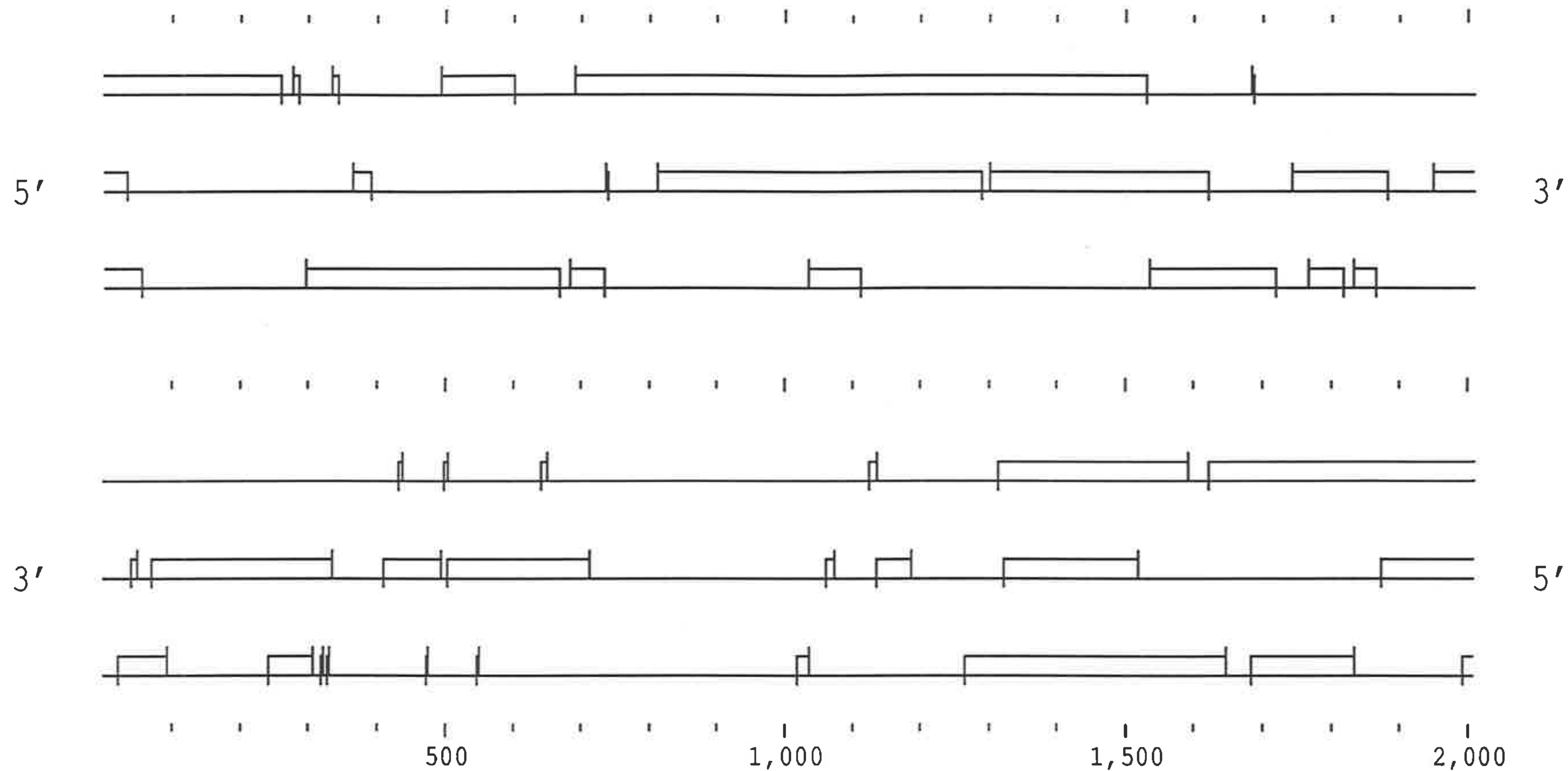


Figure 3.16 DNA sequence of pMI4N and potential avirulence gene

Both strands of pMI4N were sequenced and the 4N fragment was 2010 base pairs in length.

Open reading frame 1 is 840 base pairs in length. Universal one letter abbreviations are presented for the amino acid code.

Potential 'TATA' boxes and 'CAAT' boxes upstream from the translational start point of the gene are underlined.

Potential signals for polyadenylation downstream from the translational stop point of the gene are underlined.

```
-690  GAATTCAAAACTACCAAGGTCAGTCCGTTGTATCTGATTCAATCTGGCATTCTATCTGAC  -631

-630  TTTTCTTTTCACAGGAAGCACTGTTCGTCCACATCAAGACCTTGTGGGGTCTTTTCAAAG  -571

-570  CAAACGACGTCCCCAGCCCTCCCAATCCAGCCTTGCTGCAAGAATTTTACGCCAAATTCA  -511

-510  ATACTCCAGCGGAGGTTCAATCTGTGCTCGAAAACCCTGGCAGTGCTAATCTTGTGGCTC  -451

-450  AAAACGACATCCAAACCTTGAGGAGTCTTCGTGCCGATGGAAAGATAGGTCGTGGTATGG  -391

-390  GTAACATCGACCAGATCTACATCCTCTACATTCATGGCGTCCTAGCCAAAGTGGGTATCA  -331

-330  GAGTATGGGCTCCAGATCTGGACGACTCTCCTGACTCTCTGTACAACTCAGCCTGTCGAA  -271

-270  TCACCGCCCTCAACACATTTCGCCAACTGCTGGCAAGCGGTGCTTATAATTACATGAATA  -211

-210  TCGATCCTGAACATGTTAATTCATTTGATCACTTTGTTGCCGCTTACAATCACTACGTAC  -151

-150  ACTACTACATGGGTGACAAGTATAAGGGTGAGAAGAAGCAATCTGGTCGTGCTGCCGCCT  -91

 -90  TGAGCGCGAGGAAAACGATCCAAAAAAACCGGGAAAGGGTTAGTTCTGCATTCTTTCATC  -31

 -30  TGCTTCTCTAAATCTGCTGACTGATGCTCG ATGTTTGGTGCCTTGAAGCTCATGAAAAG  30
                                      M  F  G  A  L  K  L  M  K  R

  31  ACGTTGGACTTACCTTGATGTGAACAAGGATAAAAAGGGGTTTCCTCCTCGCTATCTGAA  90
       R  W  T  Y  L  D  V  N  K  D  K  K  G  F  P  P  R  Y  L  K

  91  GATGTGCGCCGACGTCCTAGCGCACAGTGACGATGAGCAACTTCCTGGAAAAAAATATCG  150
       M  C  A  D  V  L  A  H  S  D  D  E  Q  L  P  G  K  K  Y  R

 151  AACAATCAAAACCCTTGGTTATCGATCCAAGAACGCCAGCAAATTCTTCAGACGAATCGA  210
       T  I  K  T  L  G  Y  R  S  K  N  A  S  K  F  F  R  R  I  E

 211  AATTGAAATTGACCAGGCTGCGGTGGTCGCGGGGACCGCTAACCGCAAAAAGGAGCGCCG  270
       I  E  I  D  Q  A  A  V  V  A  G  T  A  N  R  K  K  E  R  R

 271  GCTACCAGTCACTCCAATACCTTCGCTTTTTAAGAAGGCACCAGTGGGTCTTCCAATCAA  330
       L  P  V  T  P  I  P  S  L  F  K  K  A  P  V  G  L  P  I  N

 331  CTTTTACAACACAGCATGGTATCGAAAACTCAATCCCGGTCAGAGGCGAATCATCCCCAA  390
       F  Y  N  T  A  W  Y  R  K  L  N  P  G  Q  R  R  I  I  P  N
```

391 TGCGAAAAGCGTCTGCTTTCTTCCAAATGCTGCTGAATCTTTGCTACCAGATCGTCATCC 450
A K S V C F L P N A A E S L L P D R H P

451 TGACGAACTTCTTTCAGACAAAGCGTTCAATGCCAAGTACCTCGACATACTGTCAGAACC 510
D E L L S D K A F N A K Y L D I L S E P

511 CTACAGAATACCCGAAGATGAGGAATCTGGCAGTGAAAGTGAAAGTGATTCAAATGCAGG 570
Y R I P E D E E S G S E S E S D S N A G

571 TACTAGCGAGGACTTGGAGACTGCCAATATGAATACTGACAATGAAGATGACTCAGAAAA 630
T S E D L E T A N M N T D N E D D S E N

631 TTACTCCGACGGCGATTTTGGCAATTTGTATGATGGTGATGATGTTGATGTTGAGGCCAC 690
Y S D G D F G N L Y D G D D V D V E A T

691 AGAGGAGGGGAAGGGGAAGGGGAAAGGGAAAGGGAAGATGGCCGAAGAGGATGATGACGA 750
E E G K G K G K G K G K M A E E D D D D

751 TGATGAGGAGGATGACAACTTTCTGGATGATGACGAGGATGAGGATGATGAGGAAGATGA 810
D E E D D N F L D D D E D E D D E E D D

811 TGATGATAGTGATGCGGGCATGGAAGAGGATTGAGCATGTTGACTCTGGTATTTTTTTTC 870
D D S D A G M E E D *

871 TTCTGTGTCGCTTCATTTGTCCAATTGTTTTCATTACTTATCATTTTCACTTTCTTCTTG 930

931 TTTAAAAAATACAAAAGAAAAAAAAACATGTGTGTGTCTCTGGTGAATCTTGTCCAGTTT 990

991 TTTTATGTGATTTTCGAGCTCTGTCTGTTGTCTGTGATTTGACTGCTCTTTTTTCTCTTG 1050

1051 TGATGCGTCCTAAACAAATATCTTGCAATGTTCCCCTTGACTTTTCTATTGAGTCTAAGG 1110

1111 GAGAGTTTGGACCGGGTGTGATGCCAGCAACGCATGTGTTGCTTGTCAAATTACGCAGCG 1170

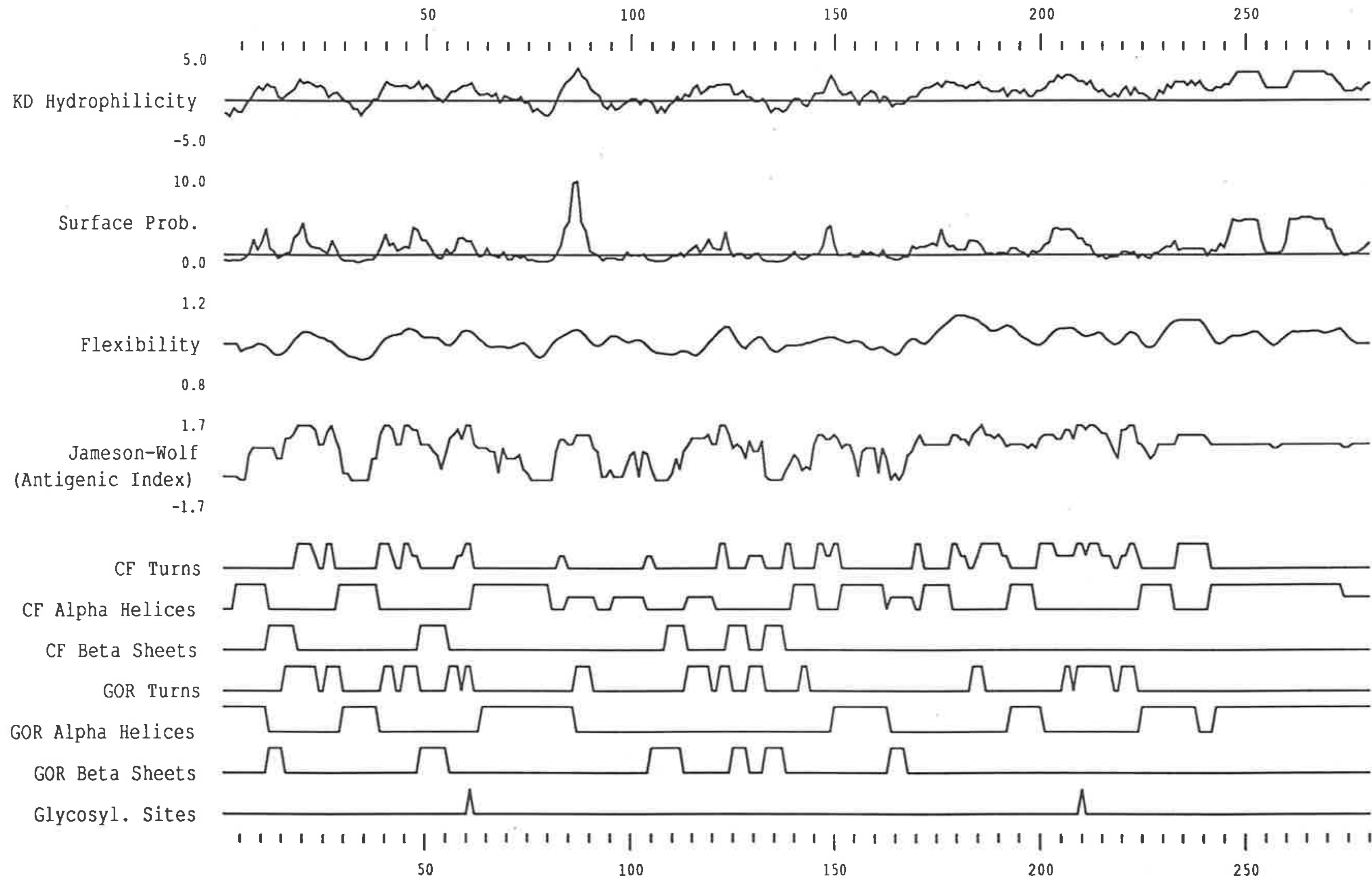
1171 GCTGGCTGAGGTTCAAGCAGTTGTAAGCAAGAACCCCAAGAGATTGTTGTTTGCCGGACT 1230

1231 GACGGTTTCCTCGCGGGTTGAACCAGTCGATGAAGAGTGAGAATACCAAATTGAGGCAAG 1290

1291 GTGGATTAGTTCTACTTTCTTCTGTGAATTC 1320

Figure 3.17 Plotstructure of potential avirulence amino acid sequence

KD Hydrophilicity - hydrophilicity according to Kyte and Doolittle (1982).
Surface Prob. - surface probability according to Emini *et al.* (1985).
Flexibility - flexibility of peptide chain according to Karplus and Schulz (Devereux *et al.*, 1984).
Jamson-Wolf Antigenic index - a measure of the probability that the region of the protein is antigenic (Devereux *et al.*, 1984).
CF Turns - residue conformations most commonly found in turns according to Chou and Fasman (1978).
CF Alpha Helices - Chou and Fasman prediction of alpha-helix forming regions that are not in conflict with other secondary structures (Chou and Fasman, 1978; Nishikawa, 1983).
CF Beta Sheets - Chou and Fasman prediction of beta-sheet structures that are not in conflict with other secondary structures (Chou and Fasman, 1978; Nishikawa, 1983).
GOR Turns - predictions of turns in protein according to Garnier-Osguthorpe-Robson (Garnier *et al.*, 1978).
GOR Alpha Helices - Garnier-Osguthorpe-Robson prediction of alpha-helix forming regions (Garnier *et al.*, 1978).
GOR Beta Sheets - Garnier-Osguthorpe-Robson prediction of beta sheet structures (Garnier *et al.*, 1978).
Glycosyl. Sites - predicted sites for glycosylation where the residues have the composition NXT or NXS (Cohen *et al.*, 1984).



PLOTSTRUCTURE of: jamie4n.p2s November 21, 1992 14:31
PEPTIDESTRUCTURE of: jamie4n.orf Ck: 297, 1 to: 280
Staden Sequence
50
100
150
200
250
5.0
KD Hydrophilicity
-5.0
10.0
Surface Prob.
0.0
1.2
Flexibility
0.8
1.7
Jameson-Wolf
(Antigenic Index)
-1.7
CF Turns
CF Alpha Helices
CF Beta Sheets
GOR Turns
GOR Alpha Helices
GOR Beta Sheets
Glycosyl. Sites
50
100
150
200
250

Figure 3.18 Predicted structure of the potential avirulence protein

This protein could be visualised spanning the membrane twice and the tail being free to move about inside or outside the cell. Predicted structure is based on plotstructure results shown in figure 3.17.

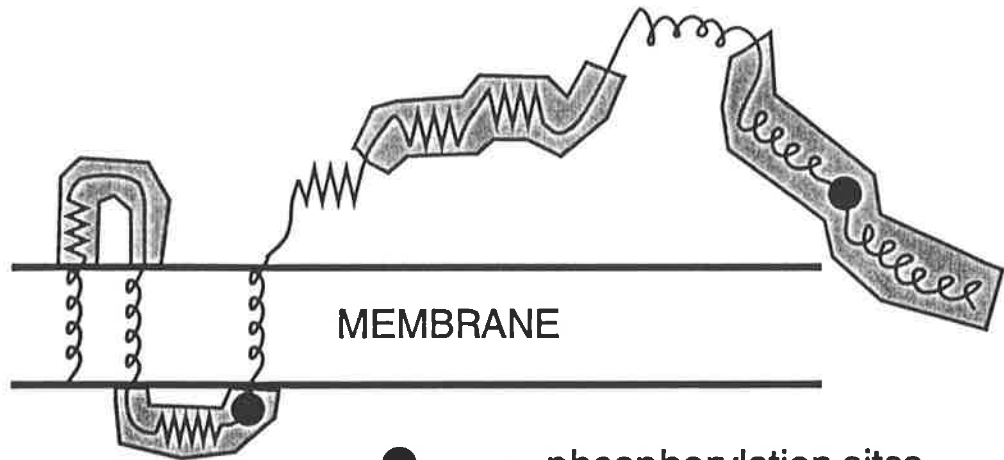

MEMBRANE
phosphorylation sites
alpha helix structures
beta sheet structures
turns
highly antigenic regions

Figure 3.19 Predicted secondary structure of the potential avirulence protein

(a) according to Chou and Fasman (1978)

(b) according to Garnier, Osguthorpe and Robson (1978)

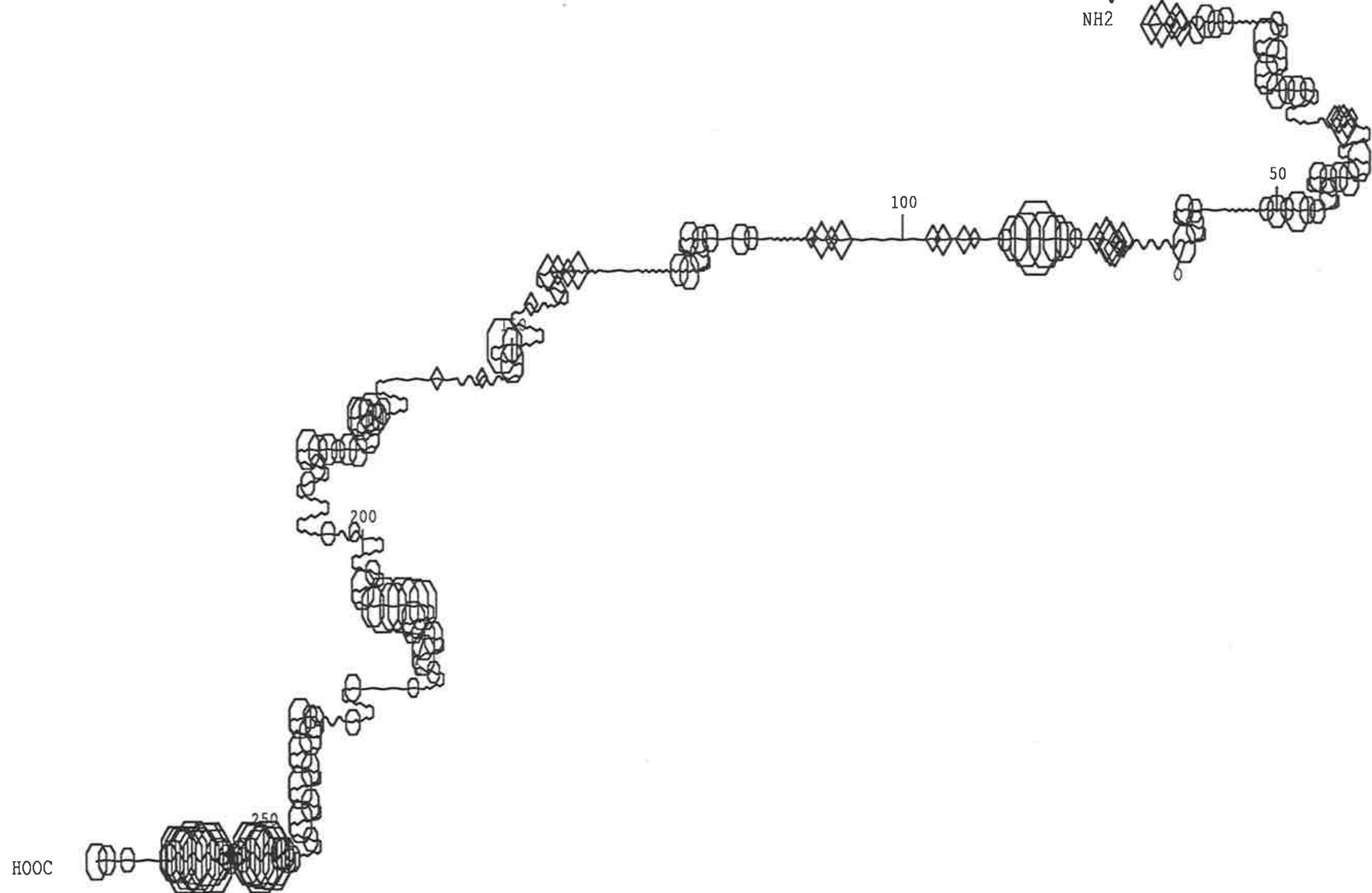
Helices are shown with a sine wave, beta sheets with a sharp saw-tooth wave, turns with 180 degrees turns and coils with a dull saw-tooth wave. Hydrophobicity is superimposed over the wave and the size of these symbols is proportional to the value of these measures. Possible glycosylation sites are marked with o (Gribskov and Devereux, 1986; Devereux, 1984).

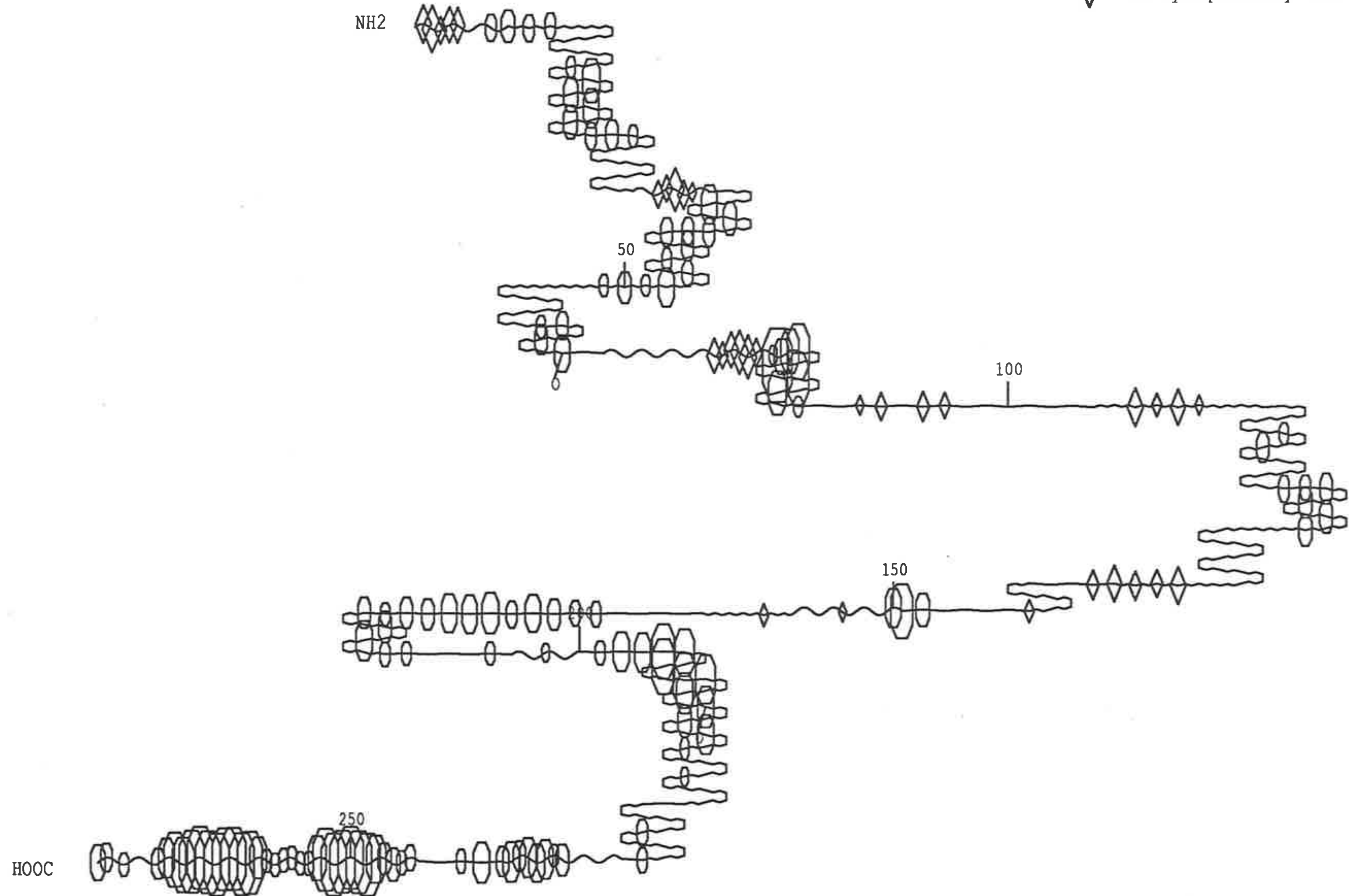
PLOTSTRUCTURE of: jamie4n.orf1 ck: 8071

Staden Sequence

Chou-Fasman Prediction
January 7, 1993 16:25

KD Hydrophilicity >=1.3
KD Hydrophobicity >=1.3




PLOTSTRUCTURE of: jamie4n.orf1 ck: 8071
Staden Sequence
Garnier-Osguthorpe-Robson Prediction
January 7, 1993 16:25
KD Hydrophilicity >=1.3
KD Hydrophobicity >=1.3
NH2
50
100
150
250
HOOC

Chapter 4
Detection and analysis of a transcript from ORF1

4.1 Northern analysis

When the rust contacts an incompatible plant, avirulence genes, if present, must be expressed to produce a product which interacts either directly or indirectly with their corresponding resistance genes to prevent infection. These avirulence genes may likewise be expressed during fungal growth in the compatible reaction of host and parasite and possibly be constitutively expressed in fungal tissues.

Northern analysis was undertaken to investigate whether an RNA transcript was detectable to determine if the potential avirulence gene was expressed during infection of plants with rust strain 228θ9θ. Total RNA was prepared from a susceptible flax variety (Hoshagabad [Hosh]) at various times after inoculation with 228θ9θ. Also, total RNA was prepared from a resistant flax variety (Birio [L6]) infected with 228θ9θ over a similar time course. Thus, one time course was made of the compatible interaction between Hosh and 228θ9θ and another one of the incompatible interaction between Birio [L6] and 228θ9θ [A-L6]. RNA from uninoculated Hosh and Birio were included as controls. RNA (10μg) from each reaction was Northern transferred onto Hybond-C extra membrane (Amersham) and hybridised with the labelled insert of pMl4N containing ORF1 and possibly other candidate avirulence genes from the A-L5, A-L6, A-L7, A-Lx group. No mRNA could be detected in either the compatible interaction (figure 4.1) or the incompatible interaction.

The Hosh-228θ9θ compatible reaction blot was subsequently probed with a piece of chloroplast DNA able to detect a specific chloroplast mRNA gene (figure 4.1). This showed that the majority of RNA was not degraded and was readily able to be detected. It was

concluded that any mRNA transcript encoded by sequences cloned in 4N could not be detected with the technique of Northern analysis due to their absence or rarity in plant-fungal RNA mixtures. Van Kan *et al.* (1991) was able to detect a mRNA transcript of *avr9* of *C. fulvum* at six days postinoculation onward using poly(A)+ RNA. The reactions assayed here were a time course ending at four days postinoculation using total RNA rather than poly(A)+ RNA. It should be considered that the RNA samples from the compatible interaction contain only a very small proportion of fungal mRNAs, especially in the early stages of infection. Even more minute amounts of fungal RNA are obtained from incompatible interactions since fungal growth is inhibited completely shortly after penetration of the plant (van Kan *et al.*, 1991)

Northern analysis of crude, total RNA appears not to be a sensitive enough method to detect a mRNA transcript encoded within pMl4N. For this reason PCR (polymerase chain reaction) analysis was considered as a potentially more sensitive method for detecting a rare transcript.

4.2: PCR analysis

Primers were designed to span the first 505 base pairs of ORF1 of pMl4N. ORF1 was originally estimated to be approximately 520 bp but subsequent sequence analysis indicated that it was 840 bp. Consequently, the 3' 340bp of the ORF was not included in PCR analyses. A primer complementary to the transcribed strand of the DNA was designed to include a *Hin*dIII restriction enzyme site: 5'-CT**AAGCTT**CGGGTATTCTGTAG-3'. This primer will allow a mRNA product of the gene to be reverse transcribed in vitro. The second primer is identical to the transcribed strand of the DNA sequence approximately 500 bp down stream of primer 1 allowing for the reverse transcription product to become double-stranded cDNA. This second primer was designed to include an *Xba*I restriction enzyme site: 5'-TG**TCTAGA**CGTTGGACTTACCT-3'. Primers were designed in areas of pMl4N

that had no sequence similarity to any other part of the subclone or other tract of LC4. Thus, the primer at the 5' end of the open reading frame starts amplification 10 codons into the open reading frame.

PCR utilised approximately 300 ng of total RNA as target template. Figure 4.2 shows that a transcription product was detected in RNA from germinated 228θ9θ rust spores (lane 2) and also in RNA from the compatible reaction between Hosh and 228θ9θ (lanes 7-10). No transcript could be detected from uninoculated Hosh (lane 6). Several controls were included to demonstrate that the PCR product originated from RNA and not contaminating DNA. Firstly, RNA was not reverse transcribed before PCR amplification (lane 3). Secondly, RNA was incubated with 0.2 µg/ul of RNase A prior to reverse transcription and amplification (lane 5). Neither of these controls yielded a PCR product. In contrast, when 228θ9θ RNA was incubated with 20 units of DNase I (RNase free) before being reverse transcribed and PCR amplified, a transcription product was detected (lane 4). These results indicated that ORF1 was a gene which was transcriptionally active in germinating spores and during growth of the rust within tissues of its compatible host plant. No PCR product could be detected in the incompatible reaction between Birio [L6] and 228θ9θ [A-L6].

Several PCR products from 228θ9θ mRNA were cloned into the plasmid vector pUBS utilising the restriction sites that were incorporated into the primers. Four clones were isolated and sequenced in both directions (figure 4.3). The sequence of clone 228θ9θpcr4 was identical to the native genomic DNA sequence of pMl4N. Clones 228θ9θpcr1 and 228θ9θpcr3 both had single base substitutions at positions 407 and 402, respectively. These were tentatively ascribed to PCR errors because no common changes were observed in any other clones i.e. the errors made by the r*Tth* polymerase which does not possess a proof-reading function. Clone 228θ9θpcr7 had single base substitutions at positions 30, 124 and 186. This level of change was considered very high in such a short stretch of sequence to assume all are PCR errors. There may therefore be a minimum of two different genes producing transcripts, one from ORF1 in pMl4N rust and one from another gene. A family of gene sequences related to each other and with different mRNA products is consistent with

the expected organisation of avirulence genes in *M. lini* (Timmis *et al.*, 1990)and also with the earlier finding that pMl4N hybridises to at least two separate *Eco*RI fragments within the rust genome.

Another rust, 271.26, is virulent (a-L5, a-L6, a-L7, a-Lx) for the four specificities (table 2.2) like the deletion mutant Aec68.1, but has previously been shown to contain sequences homologous to pERT5.8 deleted from Aec68.1 (Timmis, unpublished results). So, although both rust strains, Aec68.1 and 271.26, are virulent for these four specificities it can be assumed that in the case of 271.26, the virulence phenotype is not due to a deletion of the segment of DNA missing from Aec68.1. It is possible that virulence may be caused by a variety of other mutations which affect transcription or protein structure of the gene products.

Comparison of PCR products between a virulent rust strain and an avirulent rust strain may therefore provide confirmatory evidence that ORF1, or other sequences within the deletion, are related to pathogenicity. Consequently, PCR analysis was performed on total RNA from germinated 271.26 spores and on total RNA from a time course of the compatible reaction between Birio and 271.26. Figure 4.4 shows that transcription products which appeared were detected in germinated 271.26 rust spores and also in the compatible reaction between Birio and 271.26 and are of similar size to those of 228Ø9Ø. This result shows that ORF1 is not transcriptionally inactivated and that if ORF1 is an avirulence gene, then the virulent phenotype must be due to an altered protein product.

The 271.26 transcription products were cloned into the plasmid vector pUBS, once again utilising the restriction sites that were incorporated into the primers. Four clones were isolated and sequenced in both directions (figure 4.5). The sequence of clone 27126pcr3 was identical to that in the original DNA 228Ø9Ø genomic clone, pMl4N. Clone 27126pcr2 had a single base change at position 186. Clone 27126pcr1 had four base changes at positions 54, 80, 97 and 124. Clone 27126pcr4 had six base changes at positions 30, 80, 97, 124, 186 and 187. The changes seen at positions 80, 97, 124 and 186 were assumed to

represent different transcripts because several clones showed identical changes at these positions. Other changes were assumed, in the first instance, to be due to PCR errors due to there being no comparable changes in any other clone. Making these assumptions it was concluded that there are four different ORF1-like transcripts from different genes being produced in 271.26 rust. It is not certain that these assumptions are valid as the target mRNA was almost certainly present at very low abundance. Errors during reverse transcription or early in amplification may therefore be mistaken as evidence of transcription from a small gene family. However, the error rate (10^{-4}) is possibly not high enough to give the wide variation observed.

Consequently, the sequence of the mRNA products of ORF1 in 228Θ9Θ and 271.26 rust strains were compared (figure 4.6). It was assumed that the single base changes in 228Θ9Θpcr3 and 228Θ9Θpcr1 were due to PCR errors. It was also assumed that base change 54 in clone 27126pcr1 and base change 187 in clone 27126pcr4 were PCR errors. But all of the base changes in 228Θ9Θpcr7 had a common base change in one or more 27126pcr clones.

A significant feature of the changes observed is that the common change at position 124 results in the amino acid substitution from an arginine to a stop codon. Thus, one 228Θ9Θ transcript and two 271.26 transcripts appear to be terminated early in translation. Another interesting feature is that all of the changes observed in both the 228Θ9Θ and 271.26 transcripts only occur in the first 200 bp of the PCR products with the 300 bp of sequence toward the 3' end showing total conservation.

There appears to be no clear pattern of difference between the 228Θ9Θ transcripts and those of 271.26 indicating that the genetic control of avirulence and virulence cannot be established from these experiments. It is possible that 228Θ9Θ produces transcripts from at least two different genes and 271.26 produces transcripts from four different genes, one presumably from a gene with the 5' 500bp identical to ORF1 and the others from related genes.

What can be concluded from these experiments is that there is more variation observed

in the transcription products of ORF1-like genes in 271.26 compared with 228090. The observation of identical transcripts in both fungal strains casts some doubt on the significance of ORF1 as one of the four target avirulence genes (A-L5, A-L6, A-L7 or A-Lx). However, further PCR analysis and sequencing of RNA transcripts from the 3' 340bp of ORF1 or at its extreme 5' end may detect a clear difference between the transcripts of the two rust strains.

Figure 4.1 Hybridisation of pMI4N to total RNA

A. Formaldehyde gel showing approximately 10 µg of total RNA in each track.

Track 1 - RNA from uninfected Hosh

Track 2 - RNA from Hosh infected with 228090 for 12 hr

Track 3 - RNA from Hosh infected with 228090 for 1 day

Track 4 - RNA from Hosh infected with 228090 for 2 days

Track 5 - RNA from Hosh infected with 228090 for 4 days

B. Hybridisation of the insert from pMI4N to a Northern blot of the gel depicted in A

C. Hybridisation of a piece of chloroplast DNA able to detect chloroplast mRNA to a Northern blot of the gel dipicted in A

A

1 2 3 4 5

B

1 2 3 4 5

C

1 2 3 4 5

Figure 4.2 PCR analysis of total RNA from 228090 reactions

PCR products were electrophoresed through a 1.5% agarose gel. PCR products in lanes 2-10 underwent 2 rounds of amplification.

λ - wild type lambda DNA digested with *Hin*dIII

lane 1 - positive control supplied with the r*Tth* RNA PCR kit

lane 2 - 300 ng of total RNA from germinated 228090 spores

lane 3 - 300ng of total RNA from germinated 228090 spores not reverse transcribed but PCR amplified

lane 4 - 300 ng of total RNA from germinated 228090 spores pre-treated with DNase I

lane 5 - 300 ng of total RNA from germinated 228090 spores pre-treated with RNase A

lane 6 - 300 ng of total RNA from uninfected Hosh

lane 7 - 300 ng of total RNA from Hosh infected with 228090 for 12 hr

lane 8 - 300 ng of total RNA from Hosh infected with 228090 for 1 day

lane 9 - 300ng of total RNA from Hosh infected with 228090 for 2 days

lane 10 - 300 ng of total RNA from Hosh infected with 228090 for 4 days

lane 11 - 300 ng of DNA from germinated 228090 spores

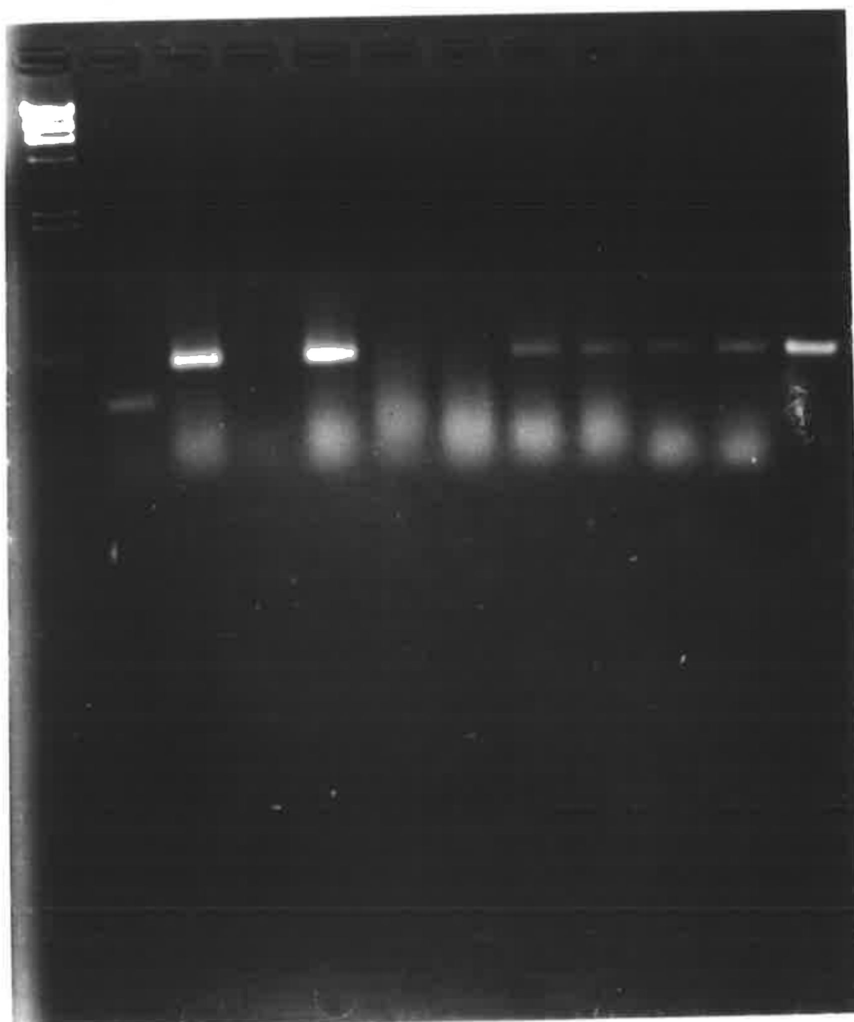

λ 1 2 3 4 5 6 7 8 9 10 11

Figure 4.3 DNA sequence of PCR products from 228090 mRNA

Four clones, 228090pcr1, 2, 4 and 7, were completely sequenced in both directions. The four clones were 505 bp in length. Any nucleotide changes observed compared to the native genomic DNA of pMl4N are underlined.

```
             1                                                  50
228090pcr4   TCTAGACGTT GGACTTACCT TGATGTGAAC AAGGATAAAA AGGGGTTTCC
228090pcr3   TCTAGACGTT GGACTTACCT TGATGTGAAC AAGGATAAAA AGGGGTTTCC
228090pcr1   TCTAGACGTT GGACTTACCT TGATGTGAAC AAGGATAAAA AGGGGTTTCC
228090pcr7   TCTAGACGTT GGACTTACCT TGATGTGAAA AAGGATAAAA AGGGGTTTCC

             51                                                100
228090pcr4   TCCTCGCTAT CTGAAGATGT GCGCCGACGT CCTAGCGCAC AGTGACGATG
228090pcr3   TCCTCGCTAT CTGAAGATGT GCGCCGACGT CCTAGCGCAC AGTGACGATG
228090pcr1   TCCTCGCTAT CTGAAGATGT GCGCCGACGT CCTAGCGCAC AGTGACGATG
228090pcr7   TCCTCGCTAT CTGAAGATGT GCGCCGACGT CCTAGCGCAC AGTGACGATG

             101                                               150
228090pcr4   AGCAACTTCC TGGAAAAAAA TATCGAACAA TCAAAACCCT TGGTTATCGA
228090pcr3   AGCAACTTCC TGGAAAAAAA TATCGAACAA TCAAAACCCT TGGTTATCGA
228090pcr1   AGCAACTTCC TGGAAAAAAA TATCGAACAA TCAAAACCCT TGGTTATCGA
228090pcr7   AGCAACTTCC TGGAAAAAAA TATTGAACAA TCAAAACCCT TGGTTATCGA

             151                                               200
228090pcr4   TCCAAGAACG CCAGCAAATT CTTCAGACGA ATCGAAATTG AAATTGACCA
228090pcr3   TCCAAGAACG CCAGCAAATT CTTCAGACGA ATCGAAATTG AAATTGACCA
228090pcr1   TCCAAGAACG CCAGCAAATT CTTCAGACGA ATCGAAATTG AAATTGACCA
228090pcr7   TCCAAGAACG CCAGCAAATT CTTCAGACGA ATCGACATTG AAATTGACCA

             201                                               250
228090pcr4   GGCTGCGGTG GTCGCGGGGA CCGCTAACCG CAAAAAGGAG CGCCGGCTAC
228090pcr3   GGCTGCGGTG GTCGCGGGGA CCGCTAACCG CAAAAAGGAG CGCCGGCTAC
228090pcr1   GGCTGCGGTG GTCGCGGGGA CCGCTAACCG CAAAAAGGAG CGCCGGCTAC
228090pcr7   GGCTGCGGTG GTCGCGGGGA CCGCTAACCG CAAAAAGGAG CGCCGGCTAC

             251                                               300
228090pcr4   CAGTCACTCC AATACCTTCG CTTTTTAAGA AGGCACCAGT GGGTCTTCCA
228090pcr3   CAGTCACTCC AATACCTTCG CTTTTTAAGA AGGCACCAGT GGGTCTTCCA
228090pcr1   CAGTCACTCC AATACCTTCG CTTTTTAAGA AGGCACCAGT GGGTCTTCCA
228090pcr7   CAGTCACTCC AATACCTTCG CTTTTTAAGA AGGCACCAGT GGGTCTTCCA

             301                                               350
228090pcr4   ATCAACTTTT ACAACACAGC ATGGTATCGA AAACTCAATC CCGGTCAGAG
228090pcr3   ATCAACTTTT ACAACACAGC ATGGTATCGA AAACTCAATC CCGGTCAGAG
228090pcr1   ATCAACTTTT ACAACACAGC ATGGTATCGA AAACTCAATC CCGGTCAGAG
228090pcr7   ATCAACTTTT ACAACACAGC ATGGTATCGA AAACTCAATC CCGGTCAGAG
```

```
             351                                                    400
228090pcr4   GCGAATCATC CCCAATGCGA AAAGCGTCTG CTTTCTTCCA AATGCTGCTG
228090pcr3   GCGAATCATC CCCAATGCGA AAAGCGTCTG CTTTCTTCCA AATGCTGCTG
228090pcr1   GCGAATCATC CCCAATGCGA AAAGCGTCTG CTTTCTTCCA AATGCTGCTG
228090pcr7   GCGAATCATC CCCAATGCGA AAAGCGTCTG CTTTCTTCCA AATGCTGCTG

             401                                                    450
228090pcr4   AATCTTTGCT ACCAGATCGT CATCCTGACG AACTTCTTTC AGACAAAGCG
228090pcr3   AGTCTTTGCT ACCAGATCGT CATCCTGACG AACTTCTTTC AGACAAAGCG
228090pcr1   AATCTTCGCT ACCAGATCGT CATCCTGACG AACTTCTTTC AGACAAAGCG
228090pcr7   AATCTTTGCT ACCAGATCGT CATCCTGACG AACTTCTTTC AGACAAAGCG

             451                                                    500
228090pcr4   TTCAATGCCA AGTACCTCGA CATACTGTCA GAACCCTACA GAATACCCGA
228090pcr3   TTCAATGCCA AGTACCTCGA CATACTGTCA GAACCCTACA GAATACCCGA
228090pcr1   TTCAATGCCA AGTACCTCGA CATACTGTCA GAACCCTACA GAATACCCGA
228090pcr7   TTCAATGCCA AGTACCTCGA CATACTGTCA GAACCCTACA GAATACCCGA

             501
228090pcr4   AGCTT
228090pcr3   AGCTT
228090pcr1   AGCTT
228090pcr7   AGCTT
```

Figure 4.4 PCR analysis of total RNA from 271.26 reactions

PCR products were electrophoresed through a 1.5% agarose gel.

λ - wild type lambda DNA digested with *Hin*dIII

lane 1 - 300 ng of total RNA from germinated 228090 spores

lane 2 - 300ng of total RNA from germinated 271.26 spores

lane 3 - 300 ng of total RNA from uninfected Birio

lane 4 - 300 ng of total RNA from Birio infected with 271.26 for 12 hr

lane 5 - 300 ng of total RNA from Birio infected with 271.26 for 1 day

lane 6 - 300 ng of total RNA from Birio infected with 271.26 for 2 days

lane 7 - 300 ng of total RNA from Birio infected with 271.26 for 4 days

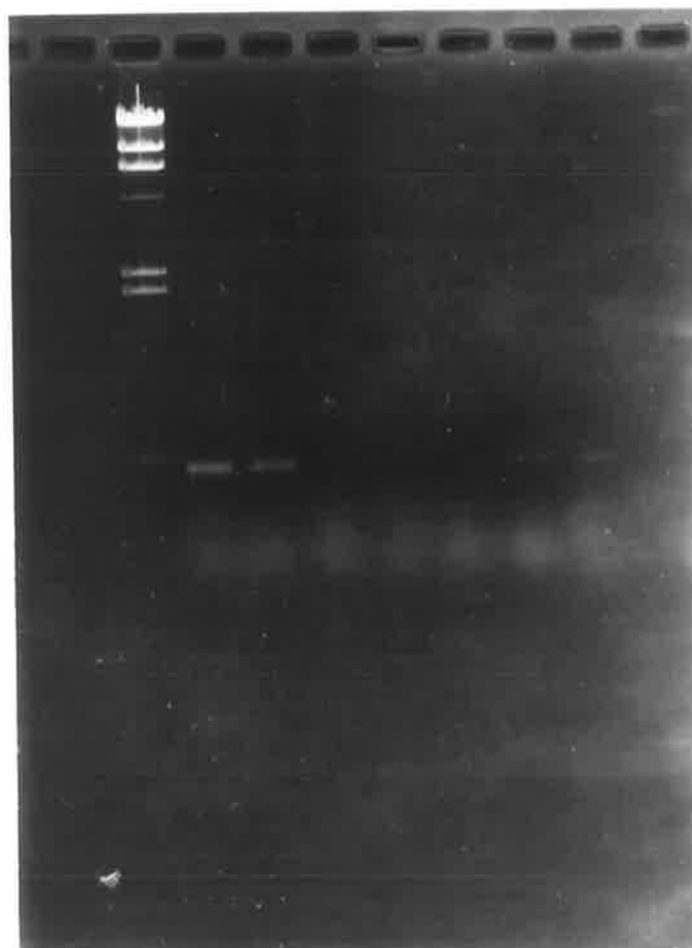

λ 1 2 3 4 5 6 7

Figure 4.5 DNA sequence of PCR products from 271.26 mRNA

Four clones, 27126pcr1, 2, 3 and 4, were completely sequenced in both directions. The four clones were 505 bp in length. Any nucleotide changes observed compared to the native genomic DNA of pMI4N are underlined.

```
            1                                                     50
27126pcr3   TCTAGACGTT GGACTTACCT TGATGTGAAC AAGGATAAAA AGGGGTTTCC
27126pcr2   TCTAGACGTT GGACTTACCT TGATGTGAAC AAGGATAAAA AGGGGTTTCC
27126pcr4   TCTAGACGTT GGACTTACCT TGATGTGAAA AAGGATAAAA AGGGGTTTCC
27126pcr1   TCTAGACGTT GGACTTACCT TGATGTGAAC AAGGATAAAA AGGGGTTTCC

            51                                                   100
27126pcr3   TCCTCGCTAT CTGAAGATGT GCGCCGACGT CCTAGCGCAC AGTGACGATG
27126pcr2   TCCTCGCTAT CTGAAGATGT GCGCCGACGT CCTAGCGCAC AGTGACGATG
27126pcr4   TCCTCGCTAT CTGAAGATGT GCGCCGACGG CCTAGCGCAC AGTGACAATG
27126pcr1   TCCCCGCTAT CTGAAGATGT GCGCCGACGG CCTAGCGCAC AGTGACAATG

            101                                                  150
27126pcr3   AGCAACTTCC TGGAAAAAAA TATCGAACAA TCAAAACCCT TGGTTATCGA
27126pcr2   AGCAACTTCC TGGAAAAAAA TATCGAACAA TCAAAACCCT TGGTTATCGA
27126pcr4   AGCAACTTCC TGGAAAAAAA TATTGAACAA TCAAAACCCT TGGTTATCGA
27126pcr1   AGCAACTTCC TGGAAAAAAA TATTGAACAA TCAAAACCCT TGGTTATCGA

            151                                                  200
27126pcr3   TCCAAGAACG CCAGCAAATT CTTCAGACGA ATCGAAATTG AAATTGACCA
27126pcr2   TCCAAGAACG CCAGCAAATT CTTCAGACGA ATCGACATTG AAATTGACCA
27126pcr4   TCCAAGAACG CCAGCAAATT CTTCAGACGA ATCGACGTTG AAATTGACCA
27126pcr1   TCCAAGAACG CCAGCAAATT CTTCAGACGA ATCGAAATTG AAATTGACCA

            201                                                  250
27126pcr3   GGCTGCGGTG GTCGCGGGGA CCGCTAACCG CAAAAAGGAG CGCCGGCTAC
27126pcr2   GGCTGCGGTG GTCGCGGGGA CCGCTAACCG CAAAAAGGAG CGCCGGCTAC
27126pcr4   GGCTGCGGTG GTCGCGGGGA CCGCTAACCG CAAAAAGGAG CGCCGGCTAC
27126pcr1   GGCTGCGGTG GTCGCGGGGA CCGCTAACCG CAAAAAGGAG CGCCGGCTAC

            251                                                  300
27126pcr3   CAGTCACTCC AATACCTTCG CTTTTTAAGA AGGCACCAGT GGGTCTTCCA
27126pcr2   CAGTCACTCC AATACCTTCG CTTTTTAAGA AGGCACCAGT GGGTCTTCCA
27126pcr4   CAGTCACTCC AATACCTTCG CTTTTTAAGA AGGCACCAGT GGGTCTTCCA
27126pcr1   CAGTCACTCC AATACCTTCG CTTTTTAAGA AGGCACCAGT GGGTCTTCCA

            301                                                  350
27126pcr3   ATCAACTTTT ACAACACAGC ATGGTATCGA AAACTCAATC CCGGTCAGAG
27126pcr2   ATCAACTTTT ACAACACAGC ATGGTATCGA AAACTCAATC CCGGTCAGAG
27126pcr4   ATCAACTTTT ACAACACAGC ATGGTATCGA AAACTCAATC CCGGTCAGAG
27126pcr1   ATCAACTTTT ACAACACAGC ATGGTATCGA AAACTCAATC CCGGTCAGAG
```

```
            351                                                    400
27126pcr3   GCGAATCATC CCCAATGCGA AAAGCGTCTG CTTTCTTCCA AATGCTGCTG
27126pcr2   GCGAATCATC CCCAATGCGA AAAGCGTCTG CTTTCTTCCA AATGCTGCTG
27126pcr4   GCGAATCATC CCCAATGCGA AAAGCGTCTG CTTTCTTCCA AATGCTGCTG
27126pcr1   GCGAATCATC CCCAATGCGA AAAGCGTCTG CTTTCTTCCA AATGCTGCTG

            401                                                    450
27126pcr3   AATCTTTGCT ACCAGATCGT CATCCTGACG AACTTCTTTC AGACAAAGCG
27126pcr2   AATCTTTGCT ACCAGATCGT CATCCTGACG AACTTCTTTC AGACAAAGCG
27126pcr4   AATCTTTGCT ACCAGATCGT CATCCTGACG AACTTCTTTC AGACAAAGCG
27126pcr1   AATCTTTGCT ACCAGATCGT CATCCTGACG AACTTCTTTC AGACAAAGCG

            451                                                    500
27126pcr3   TTCAATGCCA AGTACCTCGA CATACTGTCA GAACCCTACA GAATACCCGA
27126pcr2   TTCAATGCCA AGTACCTCGA CATACTGTCA GAACCCTACA GAATACCCGA
27126pcr4   TTCAATGCCA AGTACCTCGA CATACTGTCA GAACCCTACA GAATACCCGA
27126pcr1   TTCAATGCCA AGTACCTCGA CATACTGTCA GAACCCTACA GAATACCCGA

            501
27126pcr3   AGCTT
27126pcr2   AGCTT
27126pcr4   AGCTT
27126pcr1   AGCTT
```

Figure 4.6 Comparison of DNA sequence of PCR products from 228090 and 271.26 mRNA

Sequence of the four PCR clones from 228090, presented in figure 4.3, and the four PCR clones from 271.26, presented in figure 4.5, were compared.

Only the nucleotide changes common in more than one clone are highlighted by underlining.

Single nucleotide changes assumed to be PCR errors are not highlighted in this figure.

```
             1                                                       50
27126pcr3    TCTAGACGTT GGACTTACCT TGATGTGAAC AAGGATAAAA AGGGGTTTCC
228090pcr4   TCTAGACGTT GGACTTACCT TGATGTGAAC AAGGATAAAA AGGGGTTTCC
27126pcr2    TCTAGACGTT GGACTTACCT TGATGTGAAC AAGGATAAAA AGGGGTTTCC
228090pcr3   TCTAGACGTT GGACTTACCT TGATGTGAAC AAGGATAAAA AGGGGTTTCC
228090pcr1   TCTAGACGTT GGACTTACCT TGATGTGAAC AAGGATAAAA AGGGGTTTCC
27126pcr4    TCTAGACGTT GGACTTACCT TGATGTGAAA AAGGATAAAA AGGGGTTTCC
228090pcr7   TCTAGACGTT GGACTTACCT TGATGTGAAA AAGGATAAAA AGGGGTTTCC
27126pcr1    TCTAGACGTT GGACTTACCT TGATGTGAAC AAGGATAAAA AGGGGTTTCC

             51                                                     100
27126pcr3    TCCTCGCTAT CTGAAGATGT GCGCCGACGT CCTAGCGCAC AGTGACGATG
228090pcr4   TCCTCGCTAT CTGAAGATGT GCGCCGACGT CCTAGCGCAC AGTGACGATG
27126pcr2    TCCTCGCTAT CTGAAGATGT GCGCCGACGT CCTAGCGCAC AGTGACGATG
228090pcr3   TCCTCGCTAT CTGAAGATGT GCGCCGACGT CCTAGCGCAC AGTGACGATG
228090pcr1   TCCTCGCTAT CTGAAGATGT GCGCCGACGT CCTAGCGCAC AGTGACGATG
27126pcr4    TCCTCGCTAT CTGAAGATGT GCGCCGACGG CCTAGCGCAC AGTGACAATG
228090pcr7   TCCTCGCTAT CTGAAGATGT GCGCCGACGT CCTAGCGCAC AGTGACGATG
27126pcr1    TCCCCGCTAT CTGAAGATGT GCGCCGACGG CCTAGCGCAC AGTGACAATG

             101                                                    150
27126pcr3    AGCAACTTCC TGGAAAAAAA TATCGAACAA TCAAAACCCT TGGTTATCGA
228090pcr4   AGCAACTTCC TGGAAAAAAA TATCGAACAA TCAAAACCCT TGGTTATCGA
27126pcr2    AGCAACTTCC TGGAAAAAAA TATCGAACAA TCAAAACCCT TGGTTATCGA
228090pcr3   AGCAACTTCC TGGAAAAAAA TATCGAACAA TCAAAACCCT TGGTTATCGA
228090pcr1   AGCAACTTCC TGGAAAAAAA TATCGAACAA TCAAAACCCT TGGTTATCGA
27126pcr4    AGCAACTTCC TGGAAAAAAA TATTGAACAA TCAAAACCCT TGGTTATCGA
228090pcr7   AGCAACTTCC TGGAAAAAAA TATTGAACAA TCAAAACCCT TGGTTATCGA
27126pcr1    AGCAACTTCC TGGAAAAAAA TATTGAACAA TCAAAACCCT TGGTTATCGA

             151                                                    200
27126pcr3    TCCAAGAACG CCAGCAAATT CTTCAGACGA ATCGAAATTG AAATTGACCA
228090pcr4   TCCAAGAACG CCAGCAAATT CTTCAGACGA ATCGAAATTG AAATTGACCA
27126pcr2    TCCAAGAACG CCAGCAAATT CTTCAGACGA ATCGACATTG AAATTGACCA
228090pcr3   TCCAAGAACG CCAGCAAATT CTTCAGACGA ATCGAAATTG AAATTGACCA
228090pcr1   TCCAAGAACG CCAGCAAATT CTTCAGACGA ATCGAAATTG AAATTGACCA
27126pcr4    TCCAAGAACG CCAGCAAATT CTTCAGACGA ATCGACGTTG AAATTGACCA
228090pcr7   TCCAAGAACG CCAGCAAATT CTTCAGACGA ATCGACATTG AAATTGACCA
27126pcr1    TCCAAGAACG CCAGCAAATT CTTCAGACGA ATCGAAATTG AAATTGACCA
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            201                                                   250
27126pcr3   GGCTGCGGTG GTCGCGGGGA CCGCTAACCG CAAAAAGGAG CGCCGGCTAC
228090pcr4  GGCTGCGGTG GTCGCGGGGA CCGCTAACCG CAAAAAGGAG CGCCGGCTAC
27126pcr2   GGCTGCGGTG GTCGCGGGGA CCGCTAACCG CAAAAAGGAG CGCCGGCTAC
228090pcr3  GGCTGCGGTG GTCGCGGGGA CCGCTAACCG CAAAAAGGAG CGCCGGCTAC
228090pcr1  GGCTGCGGTG GTCGCGGGGA CCGCTAACCG CAAAAAGGAG CGCCGGCTAC
27126pcr4   GGCTGCGGTG GTCGCGGGGA CCGCTAACCG CAAAAAGGAG CGCCGGCTAC
228090pcr7  GGCTGCGGTG GTCGCGGGGA CCGCTAACCG CAAAAAGGAG CGCCGGCTAC
27126pcr1   GGCTGCGGTG GTCGCGGGGA CCGCTAACCG CAAAAAGGAG CGCCGGCTAC

            251                                                   300
27126pcr3   CAGTCACTCC AATACCTTCG CTTTTTAAGA AGGCACCAGT GGGTCTTCCA
228090pcr4  CAGTCACTCC AATACCTTCG CTTTTTAAGA AGGCACCAGT GGGTCTTCCA
27126pcr2   CAGTCACTCC AATACCTTCG CTTTTTAAGA AGGCACCAGT GGGTCTTCCA
228090pcr3  CAGTCACTCC AATACCTTCG CTTTTTAAGA AGGCACCAGT GGGTCTTCCA
228090pcr1  CAGTCACTCC AATACCTTCG CTTTTTAAGA AGGCACCAGT GGGTCTTCCA
27126pcr4   CAGTCACTCC AATACCTTCG CTTTTTAAGA AGGCACCAGT GGGTCTTCCA
228090pcr7  CAGTCACTCC AATACCTTCG CTTTTTAAGA AGGCACCAGT GGGTCTTCCA
27126pcr1   CAGTCACTCC AATACCTTCG CTTTTTAAGA AGGCACCAGT GGGTCTTCCA

            301                                                   350
27126pcr3   ATCAACTTTT ACAACACAGC ATGGTATCGA AAACTCAATC CCGGTCAGAG
228090pcr4  ATCAACTTTT ACAACACAGC ATGGTATCGA AAACTCAATC CCGGTCAGAG
27126pcr2   ATCAACTTTT ACAACACAGC ATGGTATCGA AAACTCAATC CCGGTCAGAG
228090pcr3  ATCAACTTTT ACAACACAGC ATGGTATCGA AAACTCAATC CCGGTCAGAG
228090pcr1  ATCAACTTTT ACAACACAGC ATGGTATCGA AAACTCAATC CCGGTCAGAG
27126pcr4   ATCAACTTTT ACAACACAGC ATGGTATCGA AAACTCAATC CCGGTCAGAG
228090pcr7  ATCAACTTTT ACAACACAGC ATGGTATCGA AAACTCAATC CCGGTCAGAG
27126pcr1   ATCAACTTTT ACAACACAGC ATGGTATCGA AAACTCAATC CCGGTCAGAG

            351                                                   400
27126pcr3   GCGAATCATC CCCAATGCGA AAAGCGTCTG CTTTCTTCCA AATGCTGCTG
228090pcr4  GCGAATCATC CCCAATGCGA AAAGCGTCTG CTTTCTTCCA AATGCTGCTG
27126pcr2   GCGAATCATC CCCAATGCGA AAAGCGTCTG CTTTCTTCCA AATGCTGCTG
228090pcr3  GCGAATCATC CCCAATGCGA AAAGCGTCTG CTTTCTTCCA AATGCTGCTG
228090pcr1  GCGAATCATC CCCAATGCGA AAAGCGTCTG CTTTCTTCCA AATGCTGCTG
27126pcr4   GCGAATCATC CCCAATGCGA AAAGCGTCTG CTTTCTTCCA AATGCTGCTG
228090pcr7  GCGAATCATC CCCAATGCGA AAAGCGTCTG CTTTCTTCCA AATGCTGCTG
27126pcr1   GCGAATCATC CCCAATGCGA AAAGCGTCTG CTTTCTTCCA AATGCTGCTG
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            401                                                    450
27126pcr3   AATCTTTGCT ACCAGATCGT CATCCTGACG AACTTCTTTC AGACAAAGCG
228090pcr4  AATCTTTGCT ACCAGATCGT CATCCTGACG AACTTCTTTC AGACAAAGCG
27126pcr2   AATCTTTGCT ACCAGATCGT CATCCTGACG AACTTCTTTC AGACAAAGCG
228090pcr3  AGTCTTTGCT ACCAGATCGT CATCCTGACG AACTTCTTTC AGACAAAGCG
228090pcr1  AATCTTCGCT ACCAGATCGT CATCCTGACG AACTTCTTTC AGACAAAGCG
27126pcr4   AATCTTTGCT ACCAGATCGT CATCCTGACG AACTTCTTTC AGACAAAGCG
228090pcr7  AATCTTTGCT ACCAGATCGT CATCCTGACG AACTTCTTTC AGACAAAGCG
27126pcr1   AATCTTTGCT ACCAGATCGT CATCCTGACG AACTTCTTTC AGACAAAGCG

            451                                                    500
27126pcr3   TTCAATGCCA AGTACCTCGA CATACTGTCA GAACCCTACA GAATACCCGA
228090pcr4  TTCAATGCCA AGTACCTCGA CATACTGTCA GAACCCTACA GAATACCCGA
27126pcr2   TTCAATGCCA AGTACCTCGA CATACTGTCA GAACCCTACA GAATACCCGA
228090pcr3  TTCAATGCCA AGTACCTCGA CATACTGTCA GAACCCTACA GAATACCCGA
228090pcr1  TTCAATGCCA AGTACCTCGA CATACTGTCA GAACCCTACA GAATACCCGA
27126pcr4   TTCAATGCCA AGTACCTCGA CATACTGTCA GAACCCTACA GAATACCCGA
228090pcr7  TTCAATGCCA AGTACCTCGA CATACTGTCA GAACCCTACA GAATACCCGA
27126pcr1   TTCAATGCCA AGTACCTCGA CATACTGTCA GAACCCTACA GAATACCCGA

            501
27126pcr3   AGCTT
228090pcr4  AGCTT
27126pcr2   AGCTT
228090pcr3  AGCTT
228090pcr1  AGCTT
27126pcr4   AGCTT
228090pcr7  AGCTT
27126pcr1   AGCTT
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Chapter 5
Discussion

Progress towards understanding the molecular basis of disease resistance has advanced by the molecular cloning and characterisation of a number of avirulence genes from several systems. The results described here represent first steps towards understanding the disease resistance mechanism in the flax-flax rust system at the molecular genetic level of flax rust. Initial studies have included cloning and partial characterisation of an ORF from the flax rust genome which may be one of the A-L5, A-L6, A-L7 or A-Lx avirulence gene cluster or a closely linked gene.

Nucleotide sequencing of avirulence genes has shown that avirulence activity can be accounted for by single genes and that most characterised avirulence genes encode hydrophilic proteins (Ronald *et al.*, 1992). The potential avirulence gene characterised in this study also codes for a predominantly hydrophilic polypeptide. The hydrophilic nature of the encoded proteins suggests that they are cytoplasmic with the possible exception of *avrBs3* (Bonas *et al.*, 1989) and therefore probably not directly recognised by any receptor in the plant (Jenner *et al.*, 1991). With the exception of *avr9* from *Cladosporium fulvum* (van Kan *et al.*, 1991) and *avrD* from *Pseudomonas syringae* (Kobayashi *et al.*, 1990), encoded avirulence proteins do not contain recognisable transit peptides and neither apparently does the product of ORF1 presented here when assessed by comparison with known transit peptides. If these are indeed intracellular proteins, there remains the question of how the protein functions to induce the HR. Furthermore, although some avirulence gene products have sequences in common, for the most part, avirulence genes encode dissimilar products with no consensus sequence or homology to other characterised proteins (Ronald *et al.*, 1992). Similarly ORF1 was found to have no similarity to any proteins in the computer databases searched.

Data have indicated that although the avirulence genes A-L5, A-L6, A-L7 and A-Lx

have been inactivated by deletion from Aec68.1 the DNA is still present in 271.26, this rust is not avirulent in the 4 specific host/pathogen reactions. These data are comparable with the results of Gabriel *et al.* (1986) who suggested that recessive alleles of avirulence genes occur in *X. campestris* pv. *malvacearum*. Rust strain 271.26 may contain the recessive alleles of these avirulence genes. Experiments presented here did not detect a clear difference in the sequence of the mRNAs produced by ORF1 between 228090 containing the dominant alleles for avirulence and 271.26 containing the corresponding recessive alleles for virulence.

Deletion analysis of the 3' end of *avrBs1* ORF2 and transformation studies showed that the carboxyl terminus of the protein was necessary for avirulence activity (Ronald and Staskawicz, 1988). Although several changes were observed in the 5' end of both 228090 and 271.26 PCR mRNA products, it may be that they are not important in the control of avirulence activity. Further experiments may detect a significant changes in the 3' end of ORF1 in 271.26 compared with 228090 which will present evidence to explain the difference in avirulence activity between the two rust strains. If there are changes in the gene sequence, the protein synthesised may fold incorrectly or inefficiently and, consequently, exhibit little or no biological activity. It appears that in the absence of inactivation of ORF1 at the transcriptional level there remains the possibility that, with the computer predicted N-linked glycosylation motifs, the potential avirulence protein is activated/inactivated by specific glycosylation and/or phosphorylation.

Many successful experiments have been performed using the PCR technique. But limits still exist regarding target length and sequence fidelity. Although all DNA polymerases synthesise DNA by the same basic reaction mechanism they differ in several biochemical properties. The commercially available thermostable DNA polymerases do not contain 3'-5' proofreading exonuclease activities thus having a calculated error frequency of approximately 1-1.5 x 10^{-4} (Eckert and Kunkel, 1991). The number of misincorporated nucleotides depends on the error rate during synthesis and on the number of generations.

Misincorporations occurring in an early generation of amplification are inflated in number in each subsequent cycle of doubling. The relative ease with which DNAs can be obtained by amplification should not conceal the problem that exists regarding sequence fidelity. If cloning of individual amplified fragments is required additional sequence verification is essential. Four clones from each of the 228090 and 271.26 PCR reactions were completely sequenced in both directions and probable errors and/or base substitutions were observed.

A very useful feature of the PCR is its high sensitivity. Single isolated cells or single sperm have been used for the detection of genomic target sequences (Kim and Smithies, 1988; Li *et al.*, 1988). A relatively high sensitivity has also been reported for the detectability of mRNA sequences (Vosberg, 1989). It has been shown that the RNA content of a single cell is sufficient for sequence specific amplification (Rappolee *et al.*, 1988). The low level of specific transcript observed may have contributed to the number of base changes observed. Errors early in the PCR may have been amplified through the generations. The different transcripts observed may be partly due to the inability of r*Tth* DNA polymerase to proof read but the frequency of change observed is much higher than expected from polymerase errors. The number of base substitutions observed within and between the two rust species may be a result of transcription from several related, but not identical genes.

The conventional method of detecting RNA transcripts (Northern analysis) is a much less sensitive method than PCR. This has been demonstrated in the experiments presented here. The mRNA content encoding ORF1 in 228090 and 271.26 was obviously at such a low level that Northern analysis was unable to detect the mRNA that was subsequently detected by PCR.

The PCR-based transcript identification may eventually include the analysis of complex splicing patterns. Using PCR identification of the translational stop codon in the mRNA that codes for the apolipoprotein B48 in intestinal cells has been observed. This stop codon has not been found in the respective gene. It is thought that it results from a tissue-specific co- or post-translational modification of the primary transcript (Powell *et al.*, 1987).

If the mutations leading to a change in genotype are not known (as is the case with avirulence), the PCR may provide the missing information by amplifying genomic DNA or mRNA sequences. For example, a point mutation in the HPRT gene $HPRT_{Munich}$ was verified by amplification and direct sequencing of the DNA. This mutant is a C-A transversion (Cariello *et al.*, 1988). In the gene coding for the clotting factor VIII, a previously unidentified missense mutation (a G-C transversion) has been detected by this procedure (Levinson *et al.*, 1987).

Experiments are in progress to identify and isolate the protein product of ORF1 with the use of the expression vector pGEX-2T (Smith *et al.*, 1988). At present it has not been possible to express ORF1 due to problems with obtaining a clone in the required orientation. It appears that *E. coli* hosts containing clones with the DNA sequence of ORF1 in the correct orientation and frame are not viable. The possible cause may be due to low, uninduced expression of the promoter in pGEX-2T, producing small amounts of a possibly toxic protein. This would agree with the characteristics of the *avrD* cultivar-specific elicitor from the *avrD* gene of *Pseudomonas syringae* capable of producing a HR using only minute amounts of protein (active in the nanomolar range) (Keen *et al.*, 1990).

Expression vector analysis of ORF1 may help to show if this gene is actually an avirulence gene and show whether its product is an elicitor of the HR. Analysis of the expressed protein may also elucidate the structure-function relationship by analysing the properties of normal and mutant avirulence proteins.

Genetical studies would predict four linked avirulence genes in this region of the genome and other candidate ORFs may be the target of investigations in future work. Many ORFs are present in LC4. One of possible significance is approximately the same size as ORF1 spanning the connecting ends of subclones 4N and 4B.

Linkage studies should show that these four linked avirulence genes (A-L5, A-L6, A-L7, A-Lx) are tightly linked to an RFLP observed when LC4 is used as a probe. This should provide further evidence that these genes are within the deletion of DNA from

Aec68.1.

Transformation of a virulent rust strain to confer avirulence will be the ultimate test of isolation of avirulence genes. It must be proven that the avirulence gene has actually been cloned by introducing the putative avirulence gene into a rust known to contain a susceptible allele at that avirulence-gene locus. If the transformation confers the property of avirulence to the plant, then it is clear that the given DNA segment contains the required gene.

So far, however, no transformation system is available for rust fungi although gene transformation has been successful with yeast and filamentous fungi for a number of years. Genetic transformation of yeast is now performed in a routine manner and it is only a matter of time before transformation of pathogenic fungi including rust becomes a possible procedure.

The indirect approach of identifying genes whose expression is turned on or off in the host or parasite in response to infection is a much less attractive and more time consuming approach to isolating avirulence or resistance genes. It has been shown repeatedly that the presence of a pathogen induces the expression of many genes. These genes may not be disease-specific genes, but are disease-response genes that are expressed as a result of pathogen challenge (Ellis *et al.*, 1988). The characterisation of many genes unrelated to those controlling the specific genetic interaction between host and parasite may include those expressed during wounding of the plant, as well as those directly related to microbial attack and plant defense (Lamb *et al.*, 1989). Transcripts that have been shown to accumulate during the defense response include those encoding enzymes involved in the synthesis of antimicrobial phytoalexins and lignin, hydrolytic enzymes, cell wall proteins such as hydroxyproline-rich glycoproteins, and proteinase inhibitors (Sharma *et al.*, 1992). Infact, many host defense-response genes have been cloned. These include phytoalexin biosynthesis genes such as chalcone isomerase from *Phaseolus* (Mehdy and Lamb , 1987) and resveratrol synthase from *Arachis* (Schroder *et al.*, 1988). Lignin-forming peroxidase from *Nicotiana* (Lagrimini *et al.*, 1987a,b), hydroxyproline-rich glycoproteins from *Phaseolus* (Corbin *et al.*, 1987), chitinases (Legrand *et al.*, 1987) and glucanases

(Kauffmann *et al.*, 1987) from *Nicotiana*, proteinase inhibitors from *Lycopersicon* (Graham *et al.*, 1985a,b) and pathogenesis-related proteins from *Phaseolus vulgaris* (Sharma *et al.*, 1992) comprise many of the defense-response genes cloned in recent years.

Over the last few years considerable progress has been made in understanding the key events associated with pathogen entry and attack, in defining the mechanisms involved in the elaboration of defense responses, and in the molecular cloning of avirulence genes (Lamb *et al.*, 1989). The cloning of avirulence genes has given support to the gene-for-gene hypothesis first presented by Flor (1942). At the present time the products of these various avirulence genes have not been assigned a cellular or extracellular function and it is not known how the products of the avirulence and resistance genes interact.

Complementary to the characterisation of avirulence genes is the isolation and study of resistance genes in plants. Classical genetic studies have identified dominant loci that confer resistance to many pathogens. Loci have been identified that are effective against viruses, bacteria, fungi and nematodes and these have been useful as breeding tools for disease resistance in a variety of crop species. Cloning of a resistance gene has not yet been accomplished partly because the products encoded by these loci have not been identified. It is likely that these resistance genes encode proteins involved in specific recognition of pathogens because they appear to direct a very rapid and localised induction of plant defense-response genes.

An understanding of both avirulence and resistance is necessary to further our basic knowledge of active defense in plants. It is important to isolate both avirulence genes and the resistance genes with which they interact. The isolation and characterisation of these genes will allow for investigation of the control of their expression in plants. This offers the potential for future breeding of crop plants by incorporation of new forms of disease resistance.

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