Preliminary Investigation of the Molecular Pathogenicity Determinants of *Xanthomonas campestris* pv. *zeae*

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Abstract

Xanthomonas campestris pv. *zeae* was shown to posses a vast range of potential plant cell wall degrading enzymes including at least one protease, a carboxymethylcellulase, pectin lyase, polygalacturonase and a β-endoglucanase. Replicon stability and transfer system efficiencies were determined for a range of *ori*C, and *ori*T -*tra* combinations, and suitable vectors were constructed and identified for insertional inactivation by homologous recombination. Ideal suicide replicons were found to be pACYC184 and p15A, while P, W and Q group replicons were supported by *X. campestris* pv. *zeae*. Group P and group N transfer systems were shown to be highly efficient in intra-genus matings between *Escherichia coli* and *X. campestris* pv. *zeae*, with the exception of P-*tra* systems in *trans* for the delivery of Tn5. Various cloning vectors were tested for stability and mobility. Tn5 was shown to transpose at high frequencies into the genome of the bacterial plant pathogen, and insertion was relatively random. Suitable screening assays were established to allow rapid isolation of mutants with potential virulence or pathogenic deviations, after mutagenesis. Two non-pathogenic mutants were identified, one of which was a putative *hrp*$^-$, while the other was a leaky virulence. A single mutant showing 40% reduced protease activity was also shown to exhibit reduced virulence indicating a minor role for the protease in pathogenicity. The majority of virulence mutants showed altered growth in different levels of nutritional availability and complexity. Nutritional viability (the ability to acquire and use nutrients at a sufficient rate to grow fast enough to overcome host defences) was shown to be essential for virulence and possibly pathogenicity. Wild-type *in-planta* behaviour was analysed and growth and spread patterns typical for pathogenic response identified. Chief amongst these was the requirement for a threshold level of cells per leaf area or length, before symptoms could develop. Occlusion of vascular bundles was shown not to be the primary factor in the pathogenicity of *X. campestris* pv. *zeae*. Threshold levels for lesion development indicate the absence of a diffusable lesion forming element, and possibly the requirement of cell density for induction of certain functions.

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Chapter 1 - Introduction

1.1 Determinants of Pathogenicity

Bacterial plant pathogens cause disease by virtue of there capability to enter, colonise and produce symptoms on the host plant. The ability of the organism to do this, is largely dependant on obtaining entry into the plant, surviving host defence mechanisms, obtaining sufficient nutrients to grow in the host, and doing some form of damage to the plant, typically in the form of a visible lesion and reduction in plant growth caused by tissue damage. Bacterial pathogens gain entry into the plant via a number of routes. Typically these are natural openings such as stomata or hydathodes, or transient wounds, insect or nematode vectors, or active invasion.

1.1.1 Enzymes Involved in Pathogenicity

With the exclusion of enzymes responsible for the production or modification of other macromolecular structures or toxins involved in pathogenicity or virulence, the major enzymes of relevance in pathogenicity are those exported into the environment and responsible for tissue maceration or the release of nutrients from complex macromolecular plant structures, significantly plant cell walls with their high carbohydrate content. Plant cell wall degradation is not only significant from the point of view of nutrient release for pathogen utilisation, but also plays a role in host defences, with the release of peroxidases, phenolics and activated oxygen. The major cell wall component depolymerising enzymes are found in many bacterial plant pathogens and may play a role both in penetration and in the release of nutrients from cell walls. Table 1.1 lists cell wall degrading enzymes that have been reported as produced by plant pathogens. Most cell wall depolymerising enzymes are extracellular and are usually subject to catabolic repression and substrate induction, and are thought of as fundamental to the ability of the organism to obtain nutrients in the host environment, or, in certain cases, when not within a live host, although not in any way specifying host type or range. (Walton, 1994). Numerous environmental conditions have also been shown to affect the production and export of these exoenzymes. These conditions and their effects will be discussed later.

Table 1.1 Cell-wall degrading enzymes reported from plant pathogens. After Walton (1994)

Cellulases	*B* -Xylosidase
Endo-*B* -1,4-glucanase (carboxymethylcellulase)	α -arabinofuranosidase
Cellobiohydrolase	Arabinase
B -Glucosidase	α -Glucosidase
Pectinases	*B* -1,3-Glucanase (laminarinase)
EndoPL	*B* -1,3-*B* -1,4-Glucanase
Exopolygalacturonic lyase (ExoPL)	α -galactosidase
EndoPG	*B* -galacosidase
ExoPG	*B* -1,4-galactanase
Polymethyl-esterase (PME)	Protease
B -1,4-Xylanase	Phopholipase

Of all the extracellular cell wall depolymerising enzymes produced by plant pathogens, only the cellulases, endo- and exopolygalacturonase, endopectate lyase and pectin methylesterase, and the proteases have been implicated in pathogenicity *per se*.

1.1.1.1 Cellulases

There are essentially three types of enzymes in the cellulase system (see Table 1), which appear act synergistically to release glucose from cellulose. Although chemically homogenous, the cellulose is structurally diverse, containing amorphous regions with non-associated cellulose chains, and highly ordered, crystalline regions where intra-chain and inter-chain hydrogen bonding results in the ordered structure characterised by cellulose myofibrils. These diverse ordered structures are relatively resistant to enzymatic de-polymerisation, and require the several activities to depolymerise. While endo-*B* -1,4-glucanase appears to be attack the amorphous regions exposing numerous reducing ends where cellobiohydrolase can release cellobiose for cleavage by *B* -glucosidase, this simple model does not account for the lack of evidence for a cellobiohydrolase of bacterial origin, and specifically of plant pathogenic bacterial origin (Gilbert & Hazlewood, 1993).

Despite the relative difficulty in enzymatically degrading the crystalline cellulose, cellulases (carboxymethylcellulase and *B* -glucosidase) have been reported in a number of plant pathogens. The role of the cellulases in plant pathogenicity is however questionable due to

their relatively late expression *in-planta* post infection (Cooper, 1984). In both *Pseudomonas solanacearum* and *X. campestris* pv *campestris*, endoglucanases were shown to be virulence factors (Gough *et al.* 1988; Roberts *et al.* 1988) but not essential for pathogenesis. Gough *et al.* (1988) found two endoglucanase genes, one of which coded for the major extracellular endoglucanase that was shown to play a minor role only in early stages of pathogenicity.

1.1.1.2 Pectinases

Pectinases are undoubtedly the most extensively studied of the plant cell wall degrading enzymes of bacterial plant pathogen origin. The primary reason for this is the structural susceptibility of the pectic polymers to enzymatic degradation, and the resultant tissue maceration which is characteristic of the soft rot diseases. *Erwinia chrysanthemi*, when grown in the presence of pectin, produces four or five pectin-degrading enzymes (Walton, 1994), including pectin lyase, at least five (Sauvage & Expert, 1994) and possibly as many as seven (Hugouvieux-Cotte-Pattat *et al.* 1996), pectate lyase isoenzymes and an exo-polygalacturonate lyase as well as pectin methylesterases, which collectively are responsible for the cleavage of internal glycosidic bonds and the release of oligomeric products, which are subsequently cleaved by hydrolases such as polygalacturonase and exo-poly-α-D-galacturonosidase that attack the chain termini releasing digalacturonates.

Denny *et al.* (1990) showed that *Pseudomonas solanacearum* mutants deficient in one or two polysaccharidases were still pathogenic, but had reduced virulence, the extent of which was dependant on the enzymes not being produced. Dow *et al.* (1989) showed that *X. campestris* pv *campestris* mutants deficient in one of the three polygalacturonate lyase enzymes produced black rot symptoms typical of the wild type on crucifers. As previously mentioned, the endoglucanases from *X. campestris* pv *campestris* were shown not to be essential for pathogenicity, but mutants deficient in these, although eventually producing typical symptoms, took up to 40% longer to do so (Gough *et al.*. 1988). Dow *et al.* (1987) did show complete lack of pathogenicity with polygalacturonate deficient mutants, but these results were in secretion deficient mutants, invalidating the enzyme deficiency as the primary pathogenicity factor. Extracellular pectinolytic enzymes of *Erwinia chrysanthemi* were also shown to be virulence factors, but not essential for pathogenicity (Boccara *et al.*. 1988; Roeder & Collmer, 1985).

1.1.1.3 Xylanases

The complex polysaccharide which makes up the major component of the plant cell wall hemicellulose, is systematically degraded by endo-B-1,4-xylanase, B-xylosidase, arabinofuranosidase, glucoronidase and xylan esterase, which act cooperatively (Gilbert & Hazelwood, 1993). No evidence exists for xylanases as specific pathogenicity determinants, or as major virulence factors.

1.1.1.4 Proteases

Bacterial proteases may play a role in either nutrition or cell wall component degradation for the purpose of release of further nutrients. Proteases may also be useful in overcoming plant defences such as the production of extensins which are thought to play a role in plant defence (Showalter, 1993). Tang *et* al. (1987) and Dow *et al.* (1990) showed that although not an essential pathogenecity factor, the extracellular proteolytic activity of *X. campestris* pv. *campestris*, was a major virulence factor. This evidence, together with that obtained for other plant pathogenic organisms and for other enzymes, suggests that proteases may also play a significant role in early stages of entry into and colonisation of the host plant.

1.1.1.5 Lignin Degrading Enzymes

Lignin degrading enzymes have not been implicated in plant pathogenicity, presumably due to the chemically complex and heterogenous nature of the polymer. It is however conceivable that the ability to degrade lignin could allow the bacterial pathogen to overcome certain wound responses involving lignin precursors and lignification of areas of tissue damage.

1.1.1.6 Export of Enzymes

Kang *et al.* (1994) present evidence that mutants of *Pseudomonas solanacearum* deficient in the export of extracellular proteins are severely reduced in virulence. Evidence for loss of pathogenicity by export deficient mutants of *X. campestris* (Dow *et al.* 1987) and *E. chrysanthemi* (Thurn & Chaterjee, 1985), suggests that export functions are essential for elicitation of symptoms, but this must not be seen as a function of a deficiency in extracellular enzymes, since deficiencies in the production of the enzymes do not necessarily yield the same results (see section 1.1.3). Daniels *et al.* (1984b) report loss of pathogenicity concomitant with loss of both extracellular protease and polygalacturonate lyase, indicating not only the possible significance of these enzymes, but also the likely common secretion system. Since enzymes

have been shown to be virulence factors, the complete loss of pathogenicity reported implies the secretion of additional and unknown pathogenicity factors via the same secretion system. Dow *et al.* (1987) identified mutants of *X. campestris* pv. *campestris* which were non-pathogenic and deficient only in the export of polygalacturonate. The functional enzymes were unchanged but the non-pathogenic mutant accumulated these enzymes in the periplasm. Again this is in contradiction with results obtained by Dow *et al.* (1989) and Dow *et al.* (1990) which show reduced virulence in polygalacturonate lyase deficient mutants, but not non-pathogenicity, indicating other pathogenicity determinants secreted by the same mechanism and as yet unidentified.

P. solanacearum has an outer membrane secretion system which functions once the protein has entered the periplasm by leader sequence dependant membrane transport across the plasma membrane (Huang & Schell, 1990). Salmond & Reeves (1993) describe the evidence for a type III secretion pathway in Gram-negative bacteria, and particularly plant pathogens, on the basis of the functioning of *yscA* to *yscM* of *Yersinia enterolytica* and the high levels of similarity between these genes of secretory function and *hrp* genes of *P. solanacearum*. The *pulD* family of typeII membrane traffic warden homologues include *hrpA* of *P. solanacearum*, as well as *Xps* genes of *X. campestris*, *out* genes of *Erwinia spp.* and *VirB11* of *Agrobacterium tumefaciens*. Fenselau *et* al. (1992) also showed the same similarities between *hrpA*, hrpB and hrpC of *X. campestris* pv. *vesicatoria* and *Yersinia* secretory genes. Preston *et al.* (1995) also found *ysc* homologues in an operon containing the *hrpZ* gene of *P. syringae* pvs. *syringae, glycinea* and *tomato*. This evidence, in combination with evidence for absolute requirements for secretion systems in plant pathogenic bacteria, suggests a major role in extracellular macromolecular interactions in host-pathogen interactions, a hypothesis that is being constantly refined as more evidence is obtained on the *hrp* genes of bacterial phytopathogens.

1.1.1.7 Regulation of enzymes

Regulation of the pectin degrading enzyme genes, *pelB*, *pelC* and *pelE* was shown to be environmentally regulated by low iron levels in leaf tissue (Sauvage & Expert, 1994), while anaerobosis and osmolarity have also been implicated in the expression of the *pel* genes, while growth phase and temperature, as well as nitrogen starvation have also been implicated in regulation of the *pel* cistrons (Hugouvieux-Cotte-Pattat *et al.* 1992; Hugouvieux-Cotte-Pattat

et al. 1996). A combination of regulatory factors result in differential expression of the *pel* genes with the iron regulated enzymes apparently playing a significant role in early stages of infection. Condemine & Robert-Baudouy (1995) describe coregulation of synthesis and export of virulence factors from *E. chrysanthemi* by co-regulation of the export system protein *outC*, as well as *pel* and *celZ* (a cellulase) gene expression.

Plant specific regulation of pathogenicity factors would be most energetically economical in theory. In practice, plant induced genes have been isolated from plant pathogens, supporting this postulate. Osbourn *et al.* (1987) describe the isolation of plant-induced genes by chloramphenicol acetyl transferase (CAT) assays of infected tissue with strains containing small cloned regions upstream of a promoterless CAT gene. Tn5 induced mutations were then used to identify the *dsp* genes in the cloned fragments containing plant-inducible promoters (Osbourn *et al.*. 1990). The presence of such plant inducible promoters, however, highlights one of the problems of attempting to mutate, assay for, or isolate pathogenicity factors, or any molecular determinants of bacterial plant pathogens *in-vitro*. Huang *et al.* (1995) go on to describe a complex network regulating expression of *eps* (the primary molecular pathogenicity determinant in *Pseudomonas solanacearum*) and other virulence factors including growth *in-planta*, which is dependant on multiple environmental, or plant, signals or conditions.

1.1.2 Toxins

Several bacterial plant pathogens produce diffusable substances which are toxic to the host plant. *X. albilineans*, the causative agent of leaf scald of sugarcane, produces a toxin known as albicidin, coded for by a region of the genome spanning some 69kb (Wall & Birch, 1997). No *Xanthomonas spp.* other than *X. albilineans*, have been shown to produce phytotoxins, and phytotoxins are not considered as significant determinants of pathogenicity in the genus Xanthomonas (Rudolph, 1993). This aspect of the pathogenicity of *X. campestrisd* pv. *zeae* was therefore not investigated, although the absence of diffusable substances capable of host tissue damage was demonstrated.

1.1.3 *Hrp* genes

Interactions between bacterial pathogens and plants fall into two general categories, compatible, where the bacterium grows and establishes itself in the host leading to symptom development, or, incompatible, where such growth is not observed. A distinction must be

made between basic incompatibility as a function of host species, and specific incompatibility, where specific resistance genes result in the incompatibility. In either case, the incompatible reaction often is characterised by localised necrosis, when a sufficiently high innoculum is used, referred to as the hypersensitive response (HR). In either case, the outcome of the association is dependent on a set of genes known as the hypersensitivity response/pathogenicity (*hrp*) genes (Bonas, 1994; Willis *et al.*1991; Gopalan & He, 1996).

Hypersensitivity response/pathogenicity genes of bacterial pathogens, are those genes which, by definition, are required for both pathogenicity on host plants, and the generation of the hypersensitivity response on non-host plants or host plants carrying resistance genes for which the pathogen has the appropriate avirulence (*avr*) gene. The initial description of *hrp* genes by Lindgren *et al.* (1986), was obtained by analysis of seven linked Tn5 insertions which eliminated the ability of *Pseudomonas syringae* pv *phaseolicola* to elicit HR. Six of the seven mutations in isolated mutants, prevented lesion development on inoculation into appropriate host plants and dramatically reduced *in-planta* growth. A single cosmid clone capable of complementing all these *hrp* mutations was isolated and on subsequent analysis was shown to consist of cluster approximately 22kb in length containing nine complementation groups (*hrpL, hrpAB, hrpC, hrpD, hrpE, hrpF and hrpSR*) (Rhame *et al.* 1991). *hrp* Clusters from the taxonomically related pathovars *tabaci* and *glycinea* showed a high degree of physical and functional conservation. Sequence comparisons indicate that the *hrpS* gene product may function as a regulatory protein. The fact that *hrpD*, reported to encode a membrane associated protein (Bestwick *et al.* 1995), requires the presence of a functional *hrpS*, supports the sequence similarity evidence for the regulatory function of *hrpS*. *HrpM*, has two open reading frames, coding for putative transmembrane proteins, while *hrpY* codes for a putative 80kd protein of unknown function (Mansfield *et al.* 1994).

P. syringae pv *syringae* was found to contain a cluster of 25kb composed of 16 *hrp* genes and one *hrp* associated *hrmA* gene, organised into eight transcriptional units. *HrpZ* is a plant response elicitor, P_{Pss}, which appears to be secreted via an autonomous secretion system coded for by the remainder of the *hrp* cluster. *hrpS* and *hrpR* are members of a large family of signal transduction regulatory elements, and *hrpL* appears to be an alternative sigma factor which is regulated by *hrpRS* and in turn regulates *hrpJ* and *hrpZ* production (Xiao *et al.* 1994) (see figure 1.1) as well as *avr* expression (Salmeron & Staskawicz, 1993).

The function of harpin, the specific exported HR elicitor, in the compatible reaction is unclear, but Preston *et al.* (1995) propose that leakage of sucrose and other nutrients due to alkalinization of the apoplast by *hrp*$^+$ bacteria may be beneficial to the bacterium from a nutritional point of view.

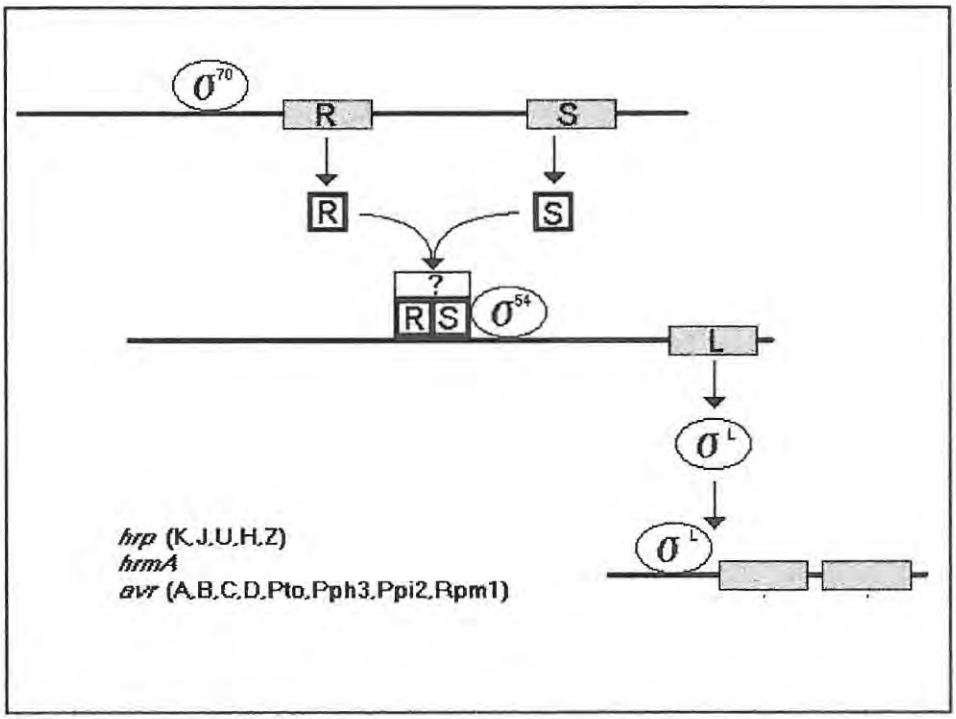

Figure1.1 Regulatory cascade controlling *hrp*, *hrmA* and *avr* expression. (After Xiao *et al.* 1994)

Expression of *hrp* genes in *P. syringae* is environmentally regulated in response to carbon and nitrogen levels and sources, pH and osmotic pressure, and in rich growth medium, fails to occur, and Xiao *et al.* (1994) postulate that in non-host plants, it is this altered nutritional environment which results in the enhanced expression of the *hrp* genes.

The *hrp* genes of the agent of southern bacterial wilt, *Pseudomonas solanacearum*, are organized into a cluster spanning approximately 26kb (Gough *et al.* 1992) containing six transcriptional units with twenty predicted proteins encoded in the cluster, eight of which belong to the group of proteins seemingly involved in Type III secretion pathway and flagellar synthesis, while an additional two appear to be related only to proteins involved in secretion systems (van Gijsegem *et al.* 1995). Trascriptional unit's one through four are required for the PopA1 protein, a genotype specific HR elicitor on petunias. Transcriptional unit's one to three code for a positive regulatory element *HrpB* (Genin *et al.* 1992) (147 amino acids) which controls the transcription of transcriptional unit's one through four and also of *hrpA*, *hrpN* and

hrpO. Of the eight putative proteins, *HrpC, HrpE, HrpI, HrpN, HrpO, HrpQ,, HrpT* and *HrpU, Hrp's C, O, T, U* and *N* are components of the flagellar basal body, with *HrpO* and *HrpN* having characteristic type III secretion system N-terminal signal sequences (Gough *et al.* 1993) *HrpQ* and *HrpE* appear to constitute a section of the flagellar motor switch apparatus at the base of the inner-membrane MS ring, while *HrpI* is an integral membrane protein which forms the base of the flagellar basal body. *HrpE* has characteristic ATPase motifs, and may therefore be associated with energy production for the flagellar apparatus, or, as postulated by van Gijsegem *et al.* (1995), may provide the necessary energy for construction of the secretion apparatus.

The *hrp* cluster of *Erwinia amylovera*, the causative agent of fire blight of pear and apple as well as various ornamental plants, spans about 40kb of which only about 22.5kb are required for the elicitation of HR on non-host plants and pathogenicity on compatible hosts. With the aid of B-glucuronidase fusions, eight transcriptional units were identified by Wei and Beer (1993). *hrpN* codes for the elicitor, harpin, which is responsible for the H^+ influx/K^+ efflux that constitutes the early HR response. The purified harpin is a 44kDa, heat stable, protease sensitive protein (Wei *et al.* 1992), which, like the harpin $_{Pss}$ coded for by *hrpZ* of *P. solanasearum* and the 34.3kDa $hrpN_{Ech}$ of *Erwinia chrysanthemi* (Bauer *et al.* 1995) is glycine rich and has a high degree of hydrophobicity. As with $harpin_{Pss}$, purified $harpin_{Ea}$ is able to induce HR, but cannot be expressed without other *hrp* genes, including *hrpI* which functions in the secretion of $harpin_{Ea}$ (Wei & Beer, 1993).

Wei *et al.* (1992) also showed that the *hrp* genes were environmentally controlled, with differential expression times and levels in host and non-host plants. In *E. amylovera*, the *hrp* genes were expressed under nitrogen deficient conditions, and repressed by glucose availability. Temperature and pH were also found to play a role in regulating the expression of the genes. This points to a relatively simple environmental regulation of the *hrp* genes and may be significant in determining the response in different host-plant environments, since the regulation of these genes could in turn regulate exoenzyme production and hence growth rate on the basis of nutrient availability, two of the major contributing virulence factors.

Investigations into the *hrp* system of *E. chrysanthemi* were hindered by copious production of the *pel* enzymes which induce a hypersensitive necrosis independantly of any *hrp* functions.

Bauer *et al.* (1994) produced Type II secretion pathway deficient mutants, which were therefore unable to secrete the pectin degrading enzymes, but which still yielded HR and allowed subsequent *hrp* gene analysis.

X. campestris pv *campestris*, the causative agent of black rot of crucifers and turnip, contains a *hrp* cluster approximately 25kb in length, which is organised into three regions controllong the HR-inducing ability and pathogenicity (Kamoun & Kado, 1990). The regulatory element of the *hrp* genes, is *hrpX*, a *hrp* gene external to the cluster observed in plant pathogenic bacteria, and found in *X. campestris* pathovars. The predicted protein of *hrpX* is a 476 amino acid molecule with a molecular mass of 52.3 kDa, with no membrane spanning domains or signal sequences (Oku *et al.* 1995). Oku *et al.* (1995) postulate a possible type III secretion pathway function for this protein, although *hrpX* does have a helix-turn-helix domain between amino acids 370 and 420 which indicates a possible DNA binding function. In either case the effect of mutations in this gene would be to reduce the plant response to the *hrp* gene-cluster products, particularly if, as has been shown for other bacterial plant pathogens, the secretion pathway is used for HR inducing elements.

As with the cluster of *E amylovera*, the *hrp* cluster of *X campestris* is environmentally regulated, and particularly, nutritionally regulated, with sucrose, glutamate and glycerol yielding highest expression levels, while peptone, casamino acids and yeast extract suppressed expression. (Kamoun & Kado, 1990). The *hrp* cluster of *X. campestris* pv. *vesicatoria* also spans a region of approximately 25kb, and consists of at least six complementation groups, designated *hrpA* to *hrpF* (Schulte and Bonas, 1992). Predicted proteins *HrpA1*, *HrpB3* and *HrpC3*, have similarity to proteins involved in secretory systems in bacterial animal pathogens.

1.1.3.1 Comparison of *hrp* clusters structures and the *hrpX* genes

1.1.3.1.1 The cluster

The commonality in *hrp* cluster size (see figure 1.2), complementation groups, transcriptional orientation and even nucleotide sequence, as well as the high level of functional similarity observed during mutational analysis, as described above, suggests a highly conserved role for these genes in the plant-pathogen interaction.

1.1.3.1.2 *hrpX*

The similarity between *hrpX* and certain animal pathogen pathogenicity determinants suggests that the postulated secretory function may be the correct one, but the sequence motifs in the putative protein suggest a regulatory rather direct implication in secretion. Of particular relevance to this question is the recent demonstration that certain bacterial periplasmic sugar binding proteins are structurally and evolutionarily related to a large class of bacterial cytoplasmic helix-turn-helix-type DNA binding proteins, many of which are repressors which bind sugar inducers (Tam & Saier, 1993) The requirement for *hrpX* for expression of other *hrp* genes, as shown by Oku *et al.* (1995) although not interpreted in this way, points to a regulatory element which may be dependant on sugar induction, as shown by Kamoun & Kado (1990) who showed sugar induction of measurable *hrp* genes from the cluster of *X. campestris* and Schulte & Bonas (1992b) who obtained similar results with pathovar *veicatoria* using sucrose as an inducer, as a means of regulating expression as a function of environmental conditions, with pathogenicity factors being expressed only *in-planta*.

Oku *et al.* (1995) describe the high level of conservation between *hrpXo* and *hrp Xc* genes. Appendix one shows the high level of similarity observed in sequences derived from *X. campestris* pv. *campestris* and *X. campestris* pv. *oryzae*. Sequence alignments with databased sequences also shows similarity to *Yersinia enterolytica* sequences involved in secretion, further complicating the issue of *hrpX* function, although the similarity between cytoplasmic DNA binding proteins and some *ysc* genes has been noted, and it is now accepted that the gene products function is essentially regulatory.

A high level of conformity has been observed with the functions of *hrp* genes where these have been elucidated. Functions that have been identified are described above. Of major significance are the recurring themes of regulation, environmental recognition - host response elicitor, and components of secretion systems. Evidence thus suggests a multifunctional group of genes responsible not only for the monitoring of environmental conditions, but the response thereto, and subsequent behaviour of the pathogen *in-planta*.

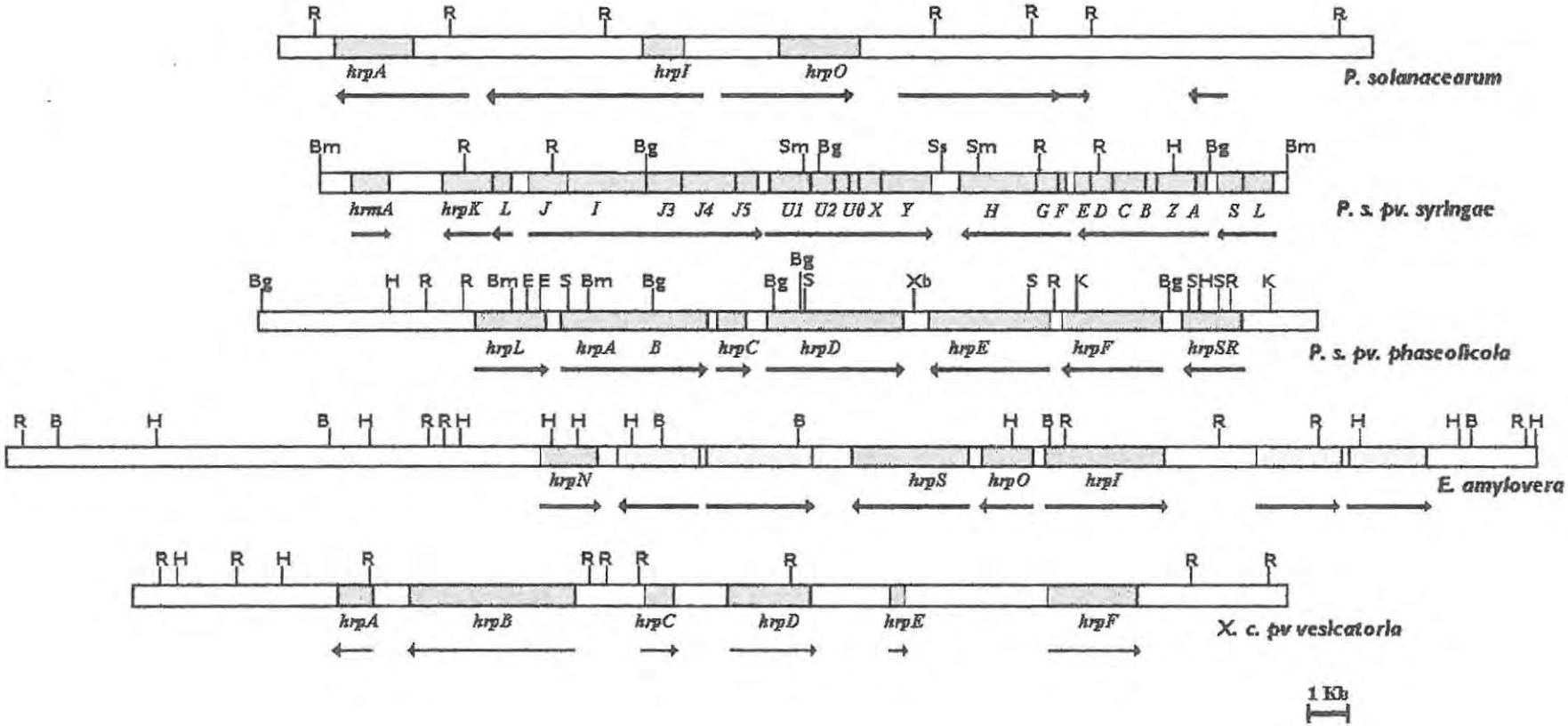

Figure 1.2 Comparison of the transcriptional organisation (arrows below gene clusters) and named complementation groups (grey blocks) of some well characterized *hrp* clusters. Letters represent restriction enzyme sites. R - *EcoRI*, B - *BamHI*, H - *HindIII*, Bg - *BglII*, S - *SmaI*, K - *KpnI*

1.1.4 *Dsp* genes

Disease specific genes (*dsp*), other than those coding for specific degradative enzymes, or those constituting the *hrp* genes, but which are essential for pathogenicity, are generally those which fulfil an essential metabolic function without which the organism in question would not be pathogenic, but could be viable either in the plant or free living. For example Hwang *et al.* (1992) isolated two open reading frames from *X. campestris* pv. *glycines* by complementation of NTG mutants with a cosmid library. The mutants, which failed to yield HR on non-host plants, were also non-pathogenic, but the complementation open reading frames were not *hrp* genes, and only limited similarity to an ion transport protein was observed for one putative protein.

Virulence functions should also be included in this group since they are by definition disease specific. Boucher *et al.* (1987) identified a region of DNA flanking the *hrp* genes of *P. solanacearum* which was involved in virulence but not hypersensitivity, and showed significant homology to sequences in eight pathovars of *X. campestris* suggesting common *dsp* factors in more distantly related bacterial phytopathogens.

Osbourn *et al.* (1990) describe mutants generated by marker exchange which showed typical *in-planta* and *in-vitro* growth characteristics as well as wild type levels of extracellular enzymes, and were non-pathogenic. Additional mutants showing the same features but with only reduced virulence were also described. In each case, the mutations were identified and the open reading frames in which the mutations occurred were compared to databases. Neither the nucleotide or putative protein sequences were homologous to any known sequences.

Tharaud *et al.* (1994) obtained *dsp*$^-$ mutants of the fire blight pathogen *Erwinia amlylovora* at a frequency of $>10^{-3}$ using phage MudIIPR13 insertion. All but one of these mutants showed rapid decrease of cell number *in-planta* post inoculation. Of particular interest was that reduced growth *in-planta* always led to absence of pathogenicity, while wild-type level growth was observed in all mutants showing reduced virulence.

Exopolysaaccharides (EPS) can be considered to be *dsp* gene products where defective mutants are shown to be non-pathogenic. EPS deficient mutants of *E. amylovera* (Tharaud *et al.* 1994), lipopolysaccharide (LPS) and EPS defective *Pseudomonas solanacearum*

(Hendrick & Sequeira, 1984 ; Kao *et al.* 1992; Huang *et al.* 1995) and *X. campestris* pv. *campestris* (Tang *et al.* 1990) have been characterised as having reduced virulence or pathogenicity. Evidence for a protective role *in-planta* exists for exopolysaccharides, and vascular occlusion by highly viscous organisms is understood to be a virulence factor (Coplin & Cook, 1990). *X. vasculorum*, the causative agent of gumming disease of sugarcane can cause leaf stripe and chlorotic leaf stripe symptoms, very similar to those of *X. campestris* pv. *zeae* in maize, as a result of xylem vessel occlusion as a function of copious EPS production. It must be noted, however, that there exists variation in the type and extent of stripes formed which appears to correlate to extracellular enzyme production, and particularly protease. Watabe *et al.* (1993) also showed that the amount and localisation of EPS produced *in-planta* by *X. oryza* pv. *oryzae*, was probably insufficient to cause occlusion of xylem vessels.

Since *dsp* genes, by definition, are detectable in their absence due to non-pathogenicity, or at least reduced virulence, potential *dsp* genes are best isolated by mutagenesis and complementation analysis.

1.1.5 Summation

Clearly the molecular determinants of pathogenicity must be divided into those which are responsible for initial host-pathogen interaction in terms of recognition and response, those which are involved in overcoming host defences, those which are responsible for growth *in-planta*, and those which are responsible for symptom development. Equally clearly, these groupings are arbitrary since growth *in-planta* is in itself a virulence factor in gummosis which is mediated by the EPS which is the pathogenicity factor. Similarly, based on the possible sugar regulation of *hrpX*, and release of sugars by enzymatic action, it is quite possible that enzyme catabolism products only available *in-planta* could actually induce *hrp* gene expression. Any number of such possibilities exist within the limitations set by existing knowledge an any attempt to categorise the molecular pathogenicity and virulence determinants must make use of hypothetical models, many of which have been proposed. The problems with such models is that they are invariably flawed through lack of sufficient knowledge to produce a detailed model. Simple models, however, can illustrate basic relationships between the components of the pathogenic response. Key points, once established in the models, provide a basis for mutational studies of molecular determinants.

Figure 1.3 shows a suggested simple model derived from available information, with key points for analysis during screening for mutant phenotypes.

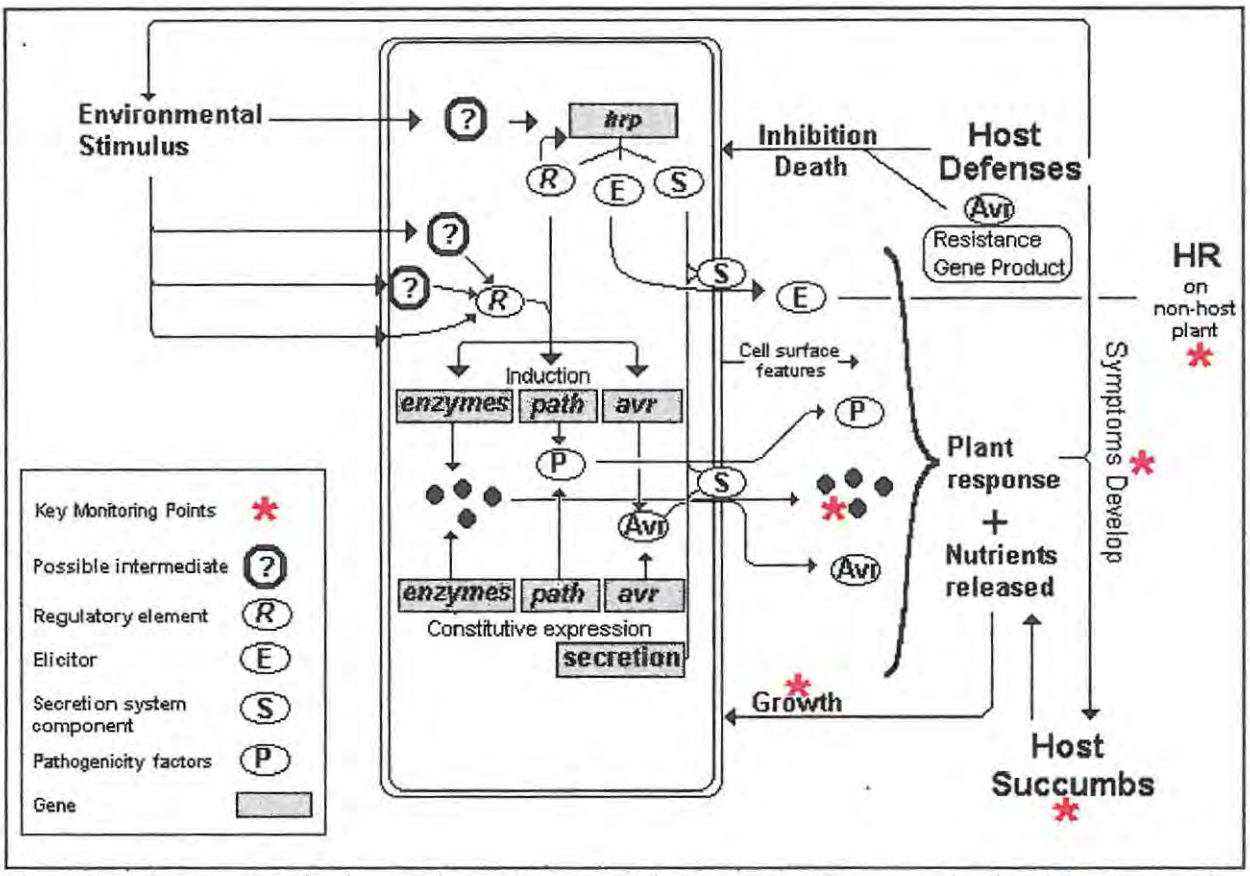

Figure 1.3 A suggested simple model showing a possible interaction between molecular determinants of pathogenicity and virulence. The model was based on all available literature (see text for details) and was drawn up as a framework for selection of screening points for mutants of *X. campestris* pv. *zeae*.

Specific incompatibility, as observed in cultivar specificity, avirulence (*Avr*), and specific resistance, although highly pertinent to host-pathogen interactions, is only of relevance where sufficient resistant cultivars have been shown to correspond to specific strains of the pathogen, and are therefore not relevant to this work and will not be discussed here. For a recent review see Parker & Coleman (1997).

Of particular interest is the recurring theme of secretion systems as pathogenicity factors, or at least the absolute requirement for secretion and the postulated secretion system function of many *hrp* genes allows the postulate that in all cases thus far studied, molecular pathogenicity factors have been produced and exported out of the cell.

1.2 *Xanthomonas campestris* pv *zeae*

Xanthomonas campestris pv *zeae* is the causative agent of bacterial leaf streak of *Zeae mays L.* (Coutihno & Wallis, 1991) and is clearly distinguishable form other pathovars capable of infecting maize (Qhobela *et al.* 1990). The disease was first reported in the Republic of South Africa in 1949 and has since not been reported in any other maize growing country. *X. campestris* pv. *zeae* is the only *Xanthomonas* spp. know to infects maize naturally; others such as *X. campestris* pv. *holcicola* and *X. campestris* pv. *vasculorum* have been shown to infect maize only by artificial inoculation. During the 1986/87 growing season, a restricted disease epiphytotic occurred in the maize growing areas of Western Transvaal, Northern Transvaal and the Orange Free State. Under these conditions, a 40% leaf tissue death was attributable to the disease. Breeding for resistance to the pathogen has been quite successful, to the point where the disease is currently not considered a major threat to the maize crop.

The pathogen, *X. campestris* pv. *zeae*, thus appears to be unique in a number ways. Firstly, in that it is the only *Xanthomonas* spp. that infects maize naturally, and secondly it appears to be restricted to the South Africa. The latter is highly improbable due to the rather frequent exchange of plant material among countries. Observations made on the relative ease of breeding for resistance indicates that the pathogen/host interaction is very specific. It may be that the pathogenicity factor produced by the pathogen is so specific, eg. phytotoxin, that the plant breeder is able to readily select for host resistance; which in turn may also be very specific. This makes this a model system for the application of mutagenesis to isolate the gene or linked genes responsible for the pathogenicity or virulence of the organism.

1.2.1 Determinants of Pathogenicity

No information exists on pathogenicity determinants of *X. campestris* pv *zeae*. Several plant pathogenic *Xanthomonas spp.* and pathovars of *X. campestris* have, however, been extensively studied and will, by virtue of their taxonomic position, serve as a basis for hypothesis since much evidence suggests interchangeability of some pathogenicity functions between pathovars (Sawczyc *et al.*. 1989) and conservation of virulence and *hrp* factors in plant pathogens in general.

1.2.1.1 Enzymes

Other than activities typically used for the identification and classification of the bacterium, no specific activities or enzymes related to plant cell wall degradation have been described for *X. campestris* pv. *zeae*. The genus *Xanthomonas* has been shown to be rich in exoglycosidases with varying and wide ranging specificities (Wong-Madden & Landry, 1995) No information on either the cellulases or pectinases of *X. campestris* pv. *zeae* was available. No specific information on the proteases of *X. campestris* pv. *zeae* was available although Coutinho & Wallis (1991) do describe casein breakdown and gelatin liquification in there work on the characterisation of the leaf streak organism. Dow *et al.* (1990) showed evidence for two proteases in *X. campestris* pv. *campestris*, a calcium-requiring serine protease and a zinc-requiring metalloprotease which were shown to be minor virulence factors. Rosato *et al.* (1994) isolated a Tn5 induced mutant of *X. campestris* pv.*campestris* which was deficient in protease production and EPS production, and was non-pathogenic. As previously stated, the apparent contradiction in the function of protease in pathogenicity is simply due to the inability of the non-pathogenic mutant to produce or export the protease because of the mutation which is also rendering it non-pathogenic.

1.2.1.2 *Hrp* genes

No information was available on the *hrp* genes of *X. campestris* pv. *zeae*. Based on extensive work on the *hrp* cluster of *X. campestris* pv. *campestris* and pathovar *vesicatoria*, a *hrp* cluster within the typical range of 23 to 28Kb was expected in *X. campestris* pv. *zeae*. *hrpXo* (*X. campestris* pv. *oryzae*) and *hrpXc* (*X. campestris* pv. *campestris*) were found to be 82% similar over 1300bp of published sequence, with regional similarity in the 3' 200bp of the sequences which approaches 90%. The relatively high level of conservation of this gene prompts the hypothesis that the gene would be present in *X. campestris* pv. *zeae* with high levels of similarity.

1.2.1.3 *Dsp* genes

No information was available on the on any *dsp* genes of *X. campestris* pv. *zeae*. A variety of pathogenicity determinants other than *hrp* genes or plant tissue degradative enzymes, and other than the EPS of *X. vasculorum*, have been identified in other *X. campestris* pathovars (Turner *et al.* 1985; Rosato *et al.* 1994; Amuthan & Mahadevan, 1994; Osbourne *et al.* 1990; Hwang *et al.* 1992, Dow *et al.* 1995) There exists sufficient evidence against vascular

occlusion as a primary mechanism of pathogenicity in monocotyledonous plants by *Xanthomonas spp.* and pathovars of *X. campestris*, on the basis of *dsp* gene identification, lack of evidence for gummosis (Watabe *et al.*. 1992) and increased striping by *X. vasculorum* with altered export or enzyme production characteristics, to hypothesise that *X. campestris* pv. *zeae* does not necessarily cause symptoms due to vascular occlusion as concluded by Coutinho & Wallis (1991).

1.2.2 Study strain

The strain used in the study (SAM116) was obtained from M Qhobela (University of Cape Town, South Africa).

1.3 Purpose of this Study

The purpose of this study was to determine which of the pathogenicity determinants mentioned in this chapter were present in *X. campestris* pv *zeae* and to obtain a minimum of information on these so as to facilitate screening of mutants generated to determine the effect of interruption of these functions. In addition to this, the major portion of the study was aimed at devising strategies for mutation of the genes responsible for these phenotypes and for the screening of such mutations in *X. campestris* pv *zeae*, and the subsequent molecular analysis of significant mutations where a substantial *in planta* variation was observed. To accomplish this, the investigation of suitable vector systems, required for routine genetic work, was included in the general aims of the study. This work, therefore, aimed at producing mutations in specific genes which had previously been shown to be significant in the pathogenicity of related organisms, and to determine the effects of such mutations on the pathogenicity of *X. campestris* pv *zeae,* or to isolate mutants showing altered pathogenicity and then determine which, if any, of the previously identified pathogenicity determinants had been effected.

Chapter 2 - Screening for the Presence of Pathogenicity/Virulence Factors in *Xanthomonas campestris* pv. *zeae*

2.1 Introduction to screening

Various enzymes involved in plant cell wall macromolecule deconstruction are produced and exported by a number of bacterial plant pathogens. The significance of these enzymes *in-planta* is, however, not clear in any of the cases where such enzymes are produced and exported. The purpose of the work described in this chapter was simply to establish which of the pertinent enzymes were in fact produced and exported by *X. campestris* pv. *zeae*. More quantifiable protease and endopectate lyase assays (being the enzymes of primary pathological significance), required for comparative analysis of mutants, were also selected.

The presence of *hrp* genes in *X. campestris* pv. *zeae* has not been established, although it seems unlikely that the typical *hrp* cluster should be absent or significantly different, or that *hrpXz*, the *hrpX* from pathovar *zeae* should be dramatically different from *hrpXc* or *hrpXo* from pathovars *campestris* or *oryzae* respectively (Kamdar *et al.* 1993). The presence of such genes would allow for their analysis in terms of function and role in pathogenicity, if, by mutagenesis such genes could be rendered non-functional. The purpose of the work represented in this chapter were simply to determine the presence of *hrp* genes in *X. campestris* pv. *zeae*, in such a manner that should *hrp*$^-$ mutants be isolated methods for the analysis of the mutation would be established, and to optimise methods for demonstrating the hypersensitive response so as to facilitate the isolation of *hrp*$^-$ mutants.

2.2 Methods and materials

SAM116 was analysed for the production and export of enzymes capable of plant cell wall macromolecule depolymerization, and the utilisation of the major storage polymer in plant tissue.

2.2.1 Plate screening

Various solid growth medium, or plate, assays were optimised for the purpose of determining which substrates could be degraded by exported enzymes. Initially, the substrates tested for degradation in solid media were, casein and gelatin, carboxymethyl cellulose (CMC), pectin, xylan and starch. All plates were tested by culturing SAM116 on the medium, and by the creation of wells in the appropriate test medium and the addition of culture supernatent, or concentrated protein fractions of the supernatent. Plates were stained and/or read daily to establish the optimum time period for assay. Culture supernatent for well based tests was obtained by removal of cells from broth cultures by centrifugation at 5000g for 10 minutes at 4°C. Supernatent was concentrated in Spectra\Por molecular porous membrane tubing (3.3ml/cm) Mw cut-off of 12,000 to 14,000, with PEG 6000. Volumes were corrected to represent comparative CFU/volume of original culture. For all substrates, cultures for supernatent tests were grown in 50% (v/v) nutrient broth and M9 minimal medium, each supplemented with 0.1%, 0.3%, 0.5%, 0.7% and 0.9% (w/v) of the relevant substrate, as well as a control containing no substrate. Whether crude or concentrated, 40µl of each sample was loaded into a well for analysis. Activity was determined by the presence of zones of substrate clearing around colonies or wells after incubation at 28°C for the appropriate length of time. All activities were confirmed by multiple colony zone analysis, and in the case of well assays, by triplicate plates each having five wells. For liquid assays, cultures and enzyme preparations were done in triplicate.

2.2.1.1 Protease activity

A standard well assay as described by Dow *et al* (1990) was used, but concentrations of skimmed milk ranging from 0.1% to 2% (w/v), and bromcresol purple concentrations ranging from zero to 0.01%, were tested in an attempt to increase the sensitivity of the assay and to achieve sharper delineation of the zones, and nutrient agar as well as Tris buffered agar (50mM, pH8) (Dow *et al.* 1990) was used as a base to provide a wider range of nutrients.

2.2.1.2 Cellulase activity

CMC was used as a substrate at concentrations of 0.5 and 0.1% for strong and weak activities respectively, and zones of hydrolysis were detected by staining with 2% congo red for 15 minutes prior to destaining with 1M NaCl for 20 minutes. Zones of activity were enhanced by flooding the destained plate with 5% (v/v) acetic acid. Reduction in nutrient content by

reduction in nutrient agar content (with appropriate addition of agar) was used to enhance sensitivity of the assay. Daniels *et al* (1988) describe an endoglucanase activity plate assay using the same principle but with the higher CMC concentration and staining with 0.1% (w/v) congo red for 30 minutes, followed by a water rinse prior to washing with 1M NaCl. This modification was also attempted for comparison. Culture supernatents for well based assays were obtained as described in section 2.2.1 above.

2.2.1.3 Pectinase activity

A plate assay was developed using pectic acid from apples in combination with various concentrations of nutrient agar, agar and pectic acid. Detection of zones was by congo red staining since congo red appears to show strong interaction with B-(1-4)-linked D-glucopyranosol units as found in the linear galactane molecule of pectic polymers. Some interaction with the remaining polymers was expected since the galacturonane polymer is a linear polymer of α1-4 linked D-galacturonanes, with the majority of remaining bonds being α1-5, and α1-3 in the low molecular mass branched chain, arabinane, and associated branches, and not expected to yield strong dye-glucan complex colour over and above the background. (Teather & Wood, 1982)

2.2.1.4 Starch hydrolysis

Amylase activity was determined by culturing the bacterium on nutrient agar supplemented with 0.1% and 1% (w/v) starch. Zones of starch hydrolysis were visualised after flooding the plates with Gram's iodine for 5 minutes.

2.2.1.5 Xylanase activity

As with the other polysaccharide substrates, zones of polymer breakdown were visualised with the congo red stain as previously described. Xylan concentrations od 0.2 and 0.5% were used.

2.2.2 Enzyme Assays

2.2.2.1 Protease assays

Protease activity was assayed using the standard TCA precipitation method with casein as a substrate. The assay was optimized using *Stenotrophomonas maltophilia,* formerly known as *Xanthomonas maltophilia* or *Pseudomonas maltophilia* (Palleroni & Bradbury, 1993)

cultured in M9 minimal medium supplemented with 1% (w/v) skimmed milk powder. At intervals during the growth of the bacteria culture, six 1.5ml samples were taken and the cells removed by centrifugation. Substrate in the form of casein was added to each microfuge tube to a final concentration of 0.5%. To three of the tubes was then added, to a final concentration of 10%, trichloroacetic acid (TCA) which was mixed immediately by inversion to act as a control against free aromatic amino acids in the growth medium. All tubes were then incubated at 30°C for 30 minutes after which a TCA precipitation step was performed on each assay tube. Absorbencies were read at 280nm, with the controls being subtracted from the final readings to give a relative protease activity.

X. campestris pv. *zeae* was then tested under the optimised assay conditions and under a wide range of culture media conditions to establish optimal conditions for the production of protease. Samples concentrated as previously described, to a 50 fold concentration were also assayed. Yeast extract, bovine serum albumen (BSA) and casein were all tested as substrates for, and to determine the effect on, induction and/or export of, the protease activity. Finally, varying concentrations of yeast extract were tested in an attempt to induce protease production by either substrate induction or amino acid starvation. Assays were performed using varying concentrations of divalent cations (Ca, Mg, Zn and Mn) and at pH values ranging from 4 to 9.

2.2.2.2 Endopectate lyase

Endopectate lyase activity was assayed for as described by Konno (1988). Both crude supernatent and concentrated supernatent was assayed. Culture conditions were as described for plate well assays. Assay mixtures containing 0.2% (w/v) acid insoluble polygalacturonate in 0.5mM $CaCl_2$, 50mM Tris-HCl (pH8.6) and enzyme extract in a volume of 1ml. Blanks consisting of pre-boiled enzyme were used for each assay to prevent enzyme extract component interference. Reactions were incubated for periods ranging from 10 minutes to one hour, and then terminated by the addition of 1ml of 100mM sodium acetate buffer at pH3.6. Terminated reactions were read at 235nm to determine the amount of unsaturated compounds. Samples concentrated as previously described, to a 50 fold concentration were also assayed.

2.2.2.3 Cellulase activities

Carboxymethylycellulase activity was not measured since the plate assay was highly effective and suitable for screening purposes. Avicellase activity was assayed for as described by Yamane & Suzuki (1988), using 0.5% (w/v) avicel in 0.1M $Na_2HPO_4.2H_2O$, 0.05M citric acid, pH7 containing the concentrated enzyme extract. Incubation was at 30°C for 12, 24 and 48 hours. Activity was determined by measurement of reducing sugars.

2.2.3 Presence of *hrp* Genes

The presence of *hrp* genes, and a method to analyse mutants for potential *hrp* gene mutation was required. To this end the postulated *hrp* gene cluster, as observed in *X. campestris* pv. *campestris*, and the *hrpX*, as observed in *X. campestris* pv. *campestris* and pathovar *vesicatoria*, were investigated.

2.2.3.1 Hypersensitive response

Tobacco, green pepper, raddish and tomato plants were tested by pressure inoculation of 200µl of a 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, and 10^9 CFU/ml mid log phase *X. campestris* pv. *zeae* cell suspension in sterile de-ionized water and saline and *E. coli* HB101 prepared in the same fashion. Pressure inoculation was performed manually using a 5ml syringe and a finger for leaf support. Inoculation in all cases was performed between major lateral veins on well established leaves, in such a way that the entire region between veins was clearly infiltrated. Where this was not that case, the inoculation of the particular suspension was repeated on another region of the leaf.

2.2.3.2 *hrp* Gene Cluster

An 8kb *EcoRI* fragment of *Pseudomonas solanacearum hrp* cluster, obtained as pBS2.17 (cloned into bluescript) from C.L Gough (Gough *et al*, 1993) (see figure2.1) was used as a probe for the *hrp* cluster of *X. campestris* pv. *zeae*.

Dot blots were performed using the DIG detection system (Boehringer Mannheim) with colorometric visualisation, as per manufacturers specifications. Dilution series of chromosomal DNA isolated from *X. campestris* pv. *zeae* overnight cultures, and *Escherichia coli* (HB101) as a control, using Flowgen's Puregene DNA isolation kit, as per manufacturers specifications, were used to establish the specificity of the cloned *hrp* cluster, in a simple dot blot experiment.

The entire plasmid and the 8kb *hrp* cluster fragment, isolated from a low melting point (Seakem Nusieve) 1% TAE agarose gel after electrophoresis of 1µg of the *EcoRI* digested plasmid, by gel extraction of the appropriate band using Promega's "PCR-prep" kit, were both used to establish the stringency required to maintain specificity.

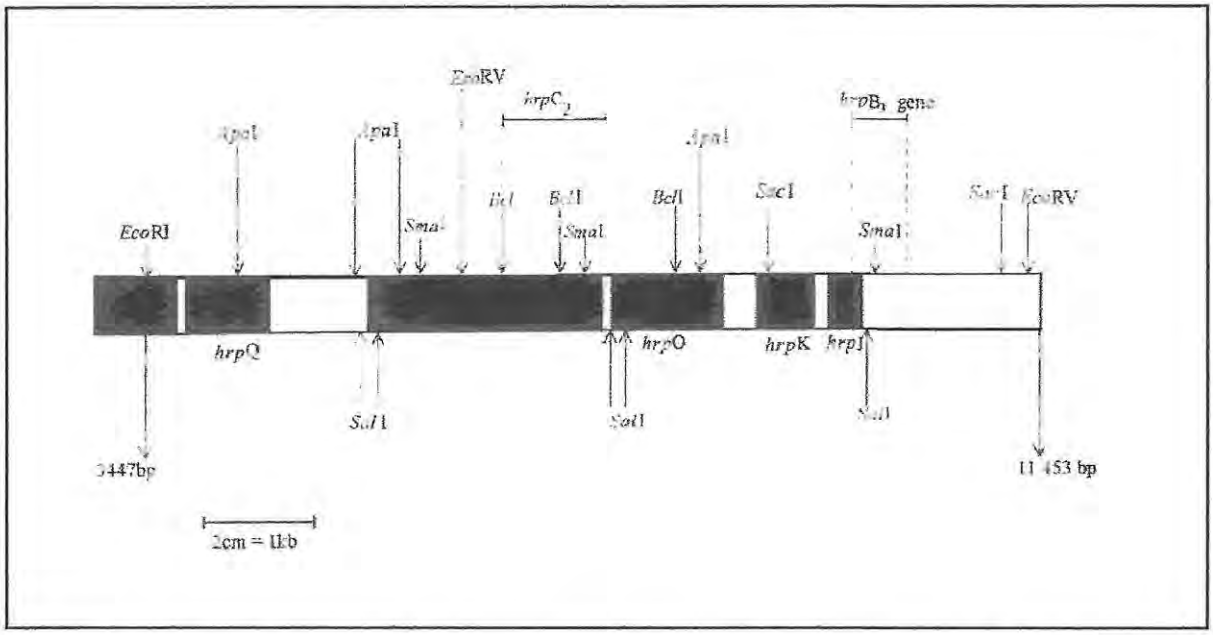

Figure 2.1 The 8Kb fragment of *Pseudomonas solanacearum*, showing areas of highest homology to sequenced *Xanthomonas hrp* genes (corresponding to the *hrpC*$_2$ and *hrpB3* genes). See appendix III for sequence alignments.

In order to enhance the signal, sequence alignments of the cloned probe and known *Xanthomonas campestris* sequences were performed in order to establish which sections of the probe had the highest potential similarity to the *hrp* cluster of pathovar *zeae*. An appropriate sequence thus identified (see figure 2.1 and appendix III) was sub-cloned in to pGEM3*zf*(-) using standard techniques (Sambrook *et al.* 1992). The specificity of the cloned sub fragment was established as before and compared to the entire fragment by direct comparison on dot blot after appropriate correction for probe length and quantity (assuming full length probes). For the preliminary analysis of the *hrp* gene cluster, isolated SAM116 chromosomal DNA was digested with the following enzymes, selected on the basis of site occurrence in *hrp* cluster of a previously characterised *X. campestris* pathovar; *EcoRI* and *HindIII*. The resultant digests were electrophoresed in 0.8% agarose with TBE at a constant voltage of 2V/cm for 20 hours. The resultant gel was blotted onto Hybond+® positively charged nylon membrane by capillary action using 0.4M NaOH as the transfer and denaturing buffer. DNA was fixed to the membrane by baking at 100°C for 30 minutes. Pre-hybridization,

hybridization and detection were done using the DIG blocking, random labeling and colourometric detection system as per manufacturers specification, in a Hybaid Micro-4 hybridazation oven at 68°C for standard hybridization buffer, or at 42°C wher DIG Easy-Hyb was used. Prehybridizations were carried out for a minimum of 3 hours and all hybridizations were overnight. Washes were performed in the hybridization oven bottles at high stringeny (68°C) unless otherwise specified.

2.2.3.3 *hrpX*

Primers were designed on the basis of regions of highest similarity between *X. campestris* pv. *campestris* and pathovar *vesicatoria* (See appendix II). The PCR was optimized on a Hybaid Omnigene thermal cycler and on the Idaho technology capillary thermal cycler using the forward primer 5'-TACTTTGCAGCGATCTCTGCG-3' and the reverse primer 5'-CTGCAGTGCGCGCCCGGTCA-3' to amplify an expected fragment of 1200bp. Degeneracy's were not used due to the high level of similarity, particularly at the 3' end. *Taq* DNA polymerase, PCR buffer and dNTP's were supplied by Boehringer Mannheim. A second forward 20mer primer was synthesized, 5'-CATGATCCTTTCGACCTACTTT-3', to overcome the low yield due to internal priming and possible primer-dimer formation. Reactions were optimized in terms of annealing temperature, $MgCl_2$ concntration, and cycle parameters. 50µl reactions containing 500ng template DNA, 200µM each dNTP, 0.5µM each primer and 0.5U *Taq* DNA polymerase and suppliers reaction buffer, in 0.5ml Safelock eppendorf tubes were used in the Hybaid machine. Reaction component concentrations were the same for capillary PCR with the exception of the addition of acetylated BSA to counter surface degradation of the enzyme, but volumes were reduced to 20µl. The amplified fragment was subjected to RFLP analysis to get an estimate of the degree of similarity between this and published sequences.

2.3 Results

Figure 2.2 shows the results obtained from the plate assays on CMC, milk powder, pectin and starch. The activities observed on each medium as zones of hydrolysis, are discussed below for each substrate.

2.3.1 Plate assay activities

2.3.1.1 Protease

1% (w/v) skimmed milk with 0.005% (w/v) bromcresol purple produced the best results in both buffered agar and nutrient agar. Faster growth on the nutrient agar based medium proved advantageous for assay purposes. Highest reproducibility was achieved after 5 days incubation at 28°C, where zones averaged 10mm in diameter with a deviation from the mean of less than 10%. The total activities obtained, as measured by zone diameter, were approximately half those obtained by Dow *et al* (1990) for *X. campestris* pv. *campestris*.

Activities obtained from culture supernatents and concentrated supernatents in well assays, correlated poorly with zones achieved on the plate assays in terms of comparable colony and pellet sizes, with the supernatent in all cases yielding much smaller zones with an average diameter of approximately 2mm as measured from the edge of the well (maximum < 4mm) for the same incubation period (two days to obtain measurable zones). Specific activities (per cell) were higher at higher cell densities. Minimal medium supplemented with casein or skimmed milk powder favoured the production of the protease activity, but in all cases concentration of the supernatent dramatically reduced the activity. The conflicting results obtained prompted more thorough induction tests using the more sensitive protease assay.

2.3.1.2 Cellulases

Optimum results were obtained using the endogluconase assay as described by Daniels *et al* (1988), with 0.2 strength nutrient agar supplemented with 1% (w/v) agar. This combination yielded clearly defined zones larger than 10mm after only two days, while comparative zones on the 0.5% CMC were only achieved after 3 days incubation. The lower congo red concentration also revealed two distinct zones of hydrolysis (see figure 2.2). The two zones were probably the result of two distinct activities. The outer zone is characterised by partial hydrolyses of the polymer, as would be affected by B-1,4-endoglucanase or carboxymethylcellulase, while the inner zone appears to represent complete removal of polymeric molecules and is therefore consistent with a cellobiohydrolase or B-glucosidase.

2.3.1.3 Pectinases

Nutrient agar containing 1% pectic acid was found to yield the best results in terms of growth of the bacteria and hence assay time, and to yield reproducible results after two days

incubation. Two zones were again discernible, with the inner zone being clearly delimited and having an average diameter of between 7 and 8mm (variations in all cases were attributable to variations in colony size as a result of inconsistent innoculum size). Unlike the inner zone on the CMC plate, clearing appears to be incomplete in the inner zone as well as the outer zone suggesting an endo- pectate lyase in the outer zone and an absence of sufficient enzymes to

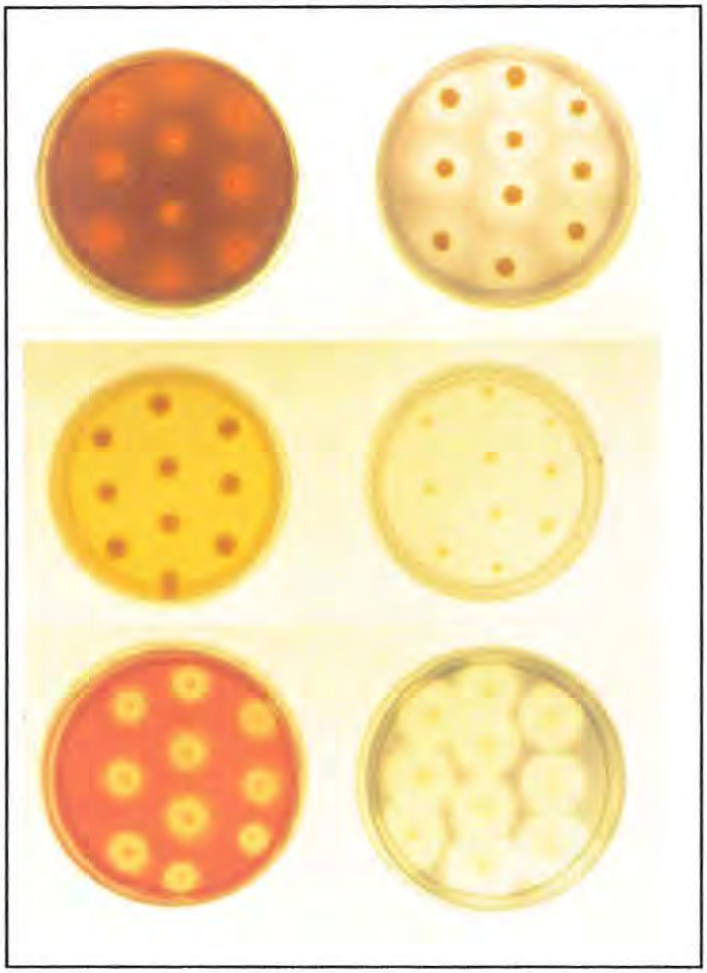

Figure 2.2 Results of plate assays where the bacteria were cultured on the medium. (top left) pectin medium (top right) milk medium (bottom left) CMC medium (bottom right) starch medium. Middle left and middle right were sucrose supplemented nutrient agar and M9 minimal medium, used to screen for EPS production and auxotrophs respectively.

completely remove polymers in the inner zone. This was, however, not entirely unexpected since the B-1,4 linked D-galactopyranose polymer, galactane, the α-1,5 linked L-arabinoruranose polymer arabinane, as well as the α-1,4 linked polygalacturonic acid would all have to be depolymerised to yield clear zones. The relatively high percentage of clearing would however suggest that the majority of polymers had been removed which may indicate

the presence of a polygalacturonic acid lyase, albeit an endo- enzyme. Assay media containing 2mM, 1mM, 0.5mM, 0.25mM, 0.125mM and 0.0625mM EDTA and EGTA respectively were used to establish the dependence of clearing of the inner zone on Ca^{2+}. Although growth was substantially inhibited by EDTA, on the 0.25mM EDTA plate, sufficient growth was observed for comparable assay, but no discernible inner zone was observed, further suggesting pectin lyase. Growth on the equivalent EGTA plate was better, and no inner zone was observed on these plates.

For each of the activities determined using well diffusion assays, higher concentrations of enzyme were detected when the organism was cultured under minimal medium conditions. No appreciable induction was however observed with the varying concentrations of substrate. Protease activities were seen to decline significantly in the concentrated fractions.

2.3.2 Enzyme assays

2.3.2.1 Protease assays

Results obtained during optimisation with *Stenotrophomonas maltophilia* are shown in figure 2.3

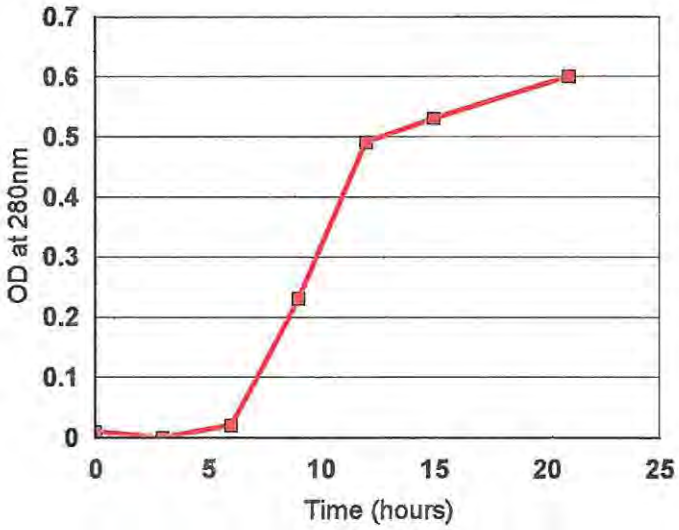

Figure 2.3 Protease activity as measured by absorbance at 280nm after exposure of casein to culture supernatent and removal of non-degraded protein by the addition of 10% TCA and centrifugation. All points are averages of three readings obtained as described in the text. Standard deviations in all cases were less than 1.5%.

On the basis of the high activities obtained, and due to activity only becoming apparent after free amino acid depletion in the medium (OD280nm dropped from 0.69 to 0.4 in medium

before induction of activity) which occurred between 5 and nine hours after inoculation, these optimised assay conditions were used on *X. campestris* pv. *zeae*. No appreciable activity was measured for SAM116 under any assay conditions with any culture or assay substrate at any concentration (data not shown). No combination of pH - divalent cation, at any of the tested concentrations yielded any activity. Electrophoresis of concentrated supernatent yielded two bands on SDS-PAGE. Sephadex G-50 separation of culture supernatents yielded two peaks absorbing at 280nm. The resultant pooled fractions also failed to yield any activity under assay conditions, abrogating any possible inhibition by contaminating proteins that may not be present in well diffusion assays due to diffusion away from wells of the protease/s. At very high cell densities in casein supplemented minimal medium, some activity was observed in crude supernatent, but not sufficient for comparative studies or analysis.

2.3.2.2 Pectinase assays

An increase in the absorbence at 235nm was observed for concentrated supernatent fractions, corresponding to the production of 1µmol unsaturated compounds (as measured off a formaldehyde standard curve). Assays on unconcentrated supernatents yielded no results after 1 hour making the assay unsuitable for routine screening. No significant variation in enzyme activity was observed with variation in substrate levels or culture media during growth of the bacterium.

2.3.2.3 Avicellase activity

A very low level of avicellase activity was observed under 0.5% CMC supplemented minimal medium culture conditions. The activity was however, extremely low.

2.3.3 *Hrp* Genes

2.3.3.1 Hypersensitive response on non-host plants

A hypersensitive response was observed in all non-host plants tested with the exception of radish. No HR induction differences on tobacco were observed between saline and water suspensions of SAM116. *E.coli* in all cases failed to yield a hypersensitive response. Figure 2.4 shows typical HR as observed on tobacco leaves after four days.

Figure 2.4 Typical HR lesions generated by pressure inoculation of 200µl SAM116 at a concentration of 10^5 CFU/ml sterile water.

Tomato plants showed the fairly typical localised chlorosis after four days but necrosis was delayed and not complete. In addition to this the chlorosis was often weak and not easily read. The minimal concentration to yield the HR was 10^4 CFU/ml. Green pepper yielded similar results but required a higher inoculum (10^5) to produce results. As with the tomato results the HR was not easily discernible or clearly defined. Tobacco yielded clearly chlorotic lesions within four days, which became rapidly necrotic thereafter. Minimal inoculum was required to generate this response, but reproducibility and consistency of HR appearance was enhanced by using 10^5 CFU/ml.

2.3.3.2 *hrp* Genes other than *hrpX*

Dot blot analysis of SAM116 at high stringency, using the entire 8kb *hrp* cluster fragment of *P. solanacearum* produced no signal in any DNA tested other than the vector pBS2.17, which was used as a positive control. Lower stringency washes, resulted in positive tests for SAM116 but also for *E.coli* and *X. maltophilia* which were used as negative controls. Hybridizations at 55, 60, 62 and 62°C all yielded non specific results with low stringency washes, while hybridization temperatures over 64°C produced signal only from the positive control, even at low stringency washes. In an attempt to increase the specificity of the hybridization, but allow for reduced stringency, an internal fragment of the *P. solanacearum* clone showing highest similarity to any *Xanthomonas* hrp gene sequences, was excised, purified by gel purification using Promega's PCR-prep kit, and labelled using DIG HI-Prime labelling kit (Boehringer Mannheim). A reasonable signal was observed with this probe in one

experiment using Boehringer Mannheim's Easy-Hyb system as per manufacturers specifications with hybridization at 42°C and washing at room temperature (See figure 2.5)

Using the conditions as identified to give faint but specific signal in the dot blot experiment, a Southern was performed as described. One band was observed in addition to those present in the positive controls, pBS2.17 and *PstI* digested pBS2.17, but the band was weak and of an unexpected size and this result could not be reproduced in three subsequent Southern blots.

Figure 2.5 Dot blot performed on positive, negative and test organisms using the 3.7Kb internal *SmaI* fragment of the 8Kb cloned fragment of the *P. solanacearum hrp* cluster. Hybridizations were performed on positively charged nylon membrane at 42°C in a Hybaid Micro-4 hybridization oven, with post hybridization stringency washes performed at room temperature, using Boehringer Mannheims's Easy-hyb DIG labeling and detection system as per manufacturers specifications. Rows 1 and 2 are dilutions with row 2 representing 50% of the DNA in row 1. All chromosomal DNA was at concentrations of 1μg and 500ng, while plasmid DNA was at 200 and 100ng. Column (A) contained pBS2.17, column B was *X. maltophilia* total DNA, column (C) was *X. campestris*pv. *zeae* total DNA and column (D) was *E. coli* total DNA.

The author is however confident that use of a more sensitive detection system (such as chemiluminescence enzyme substrates or P^{32} labelling) and the use of additional probes from *Xanthomonas campestris* pathovars would yield information not only the presence, but also on the size and degree of similarity to other *hrp* clusters, and that the poor results obtained were simply a combination of low sensitivity and relatively low similarity.

2.3.3.3 Amplification of *hrpX*

Results of optimized amplification using the first two primers can be seen in figure 2.6. Irrespective of PCR parameters, the predominant, and under stringent annealing conditions the only, PCR product was a 310bp fragment. A potential secondary annealing site was identified in the *hrpXo* sequence, prompting the construction of an alternative forward primer. Subsequent amplification with the new primer combination, despite extensive optimization of

$MgCl_2$, template and primer concentrations, and annealing temperatures between 45 and 65°C, again yielded a predominance of a smaller fragment, but did also produce a fragment within the expected size range of 1200 to 1300 bp. (See figure 2.7) Taking into consideration the relative sizes and positions of the two primer - fragment size combinations of the smaller fragments, it seems likely that the reverse primer was annealing at an internal site. During an attempt to optimise annealing temperature for the primer pair, highly reproducible smaller fragments were observed (See figure 2.8). At no annealing temperature did the smaller bands disappear in favour of the band of predicted size

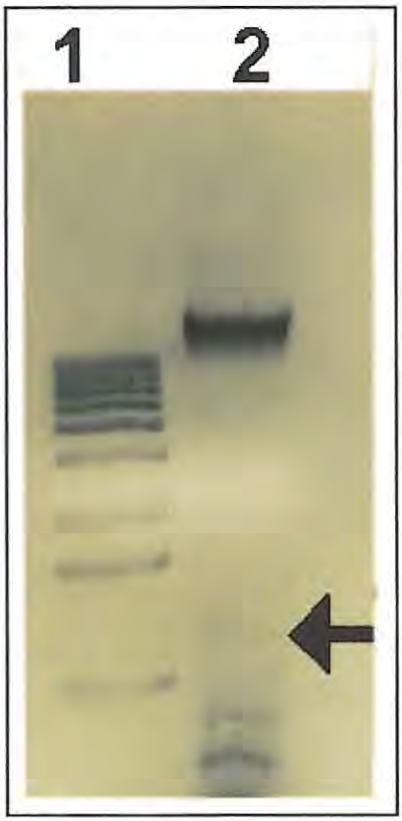

Figure 2.6 Negative image of ethidium bromide stained 1% agarose gel. Amplification products obtained with an annealing temperature of 55°C for 20 seconds, 1 minutes extension and 30 seconds denaturation in a Hybaid Omnigene thermal cycler. *hrp* foward primer 5'-TGGAGTGCGGGCCCCGGTGGT-3' and the original reverse primer 5'-CTGCAGTGCGCGCCCGGTCA-3', at 100μM each. 100μM of each dNTP, 1,5mM $MgCl_2$ and 1U*Taq* were used in a 50μl reaction with 400ng template DNA. Lane 1 is a molecular weight marker and lane 2 the amplification product. The arrow indicates an amplification product of the appropriate size. Note the poor yield and many smaller fragments.

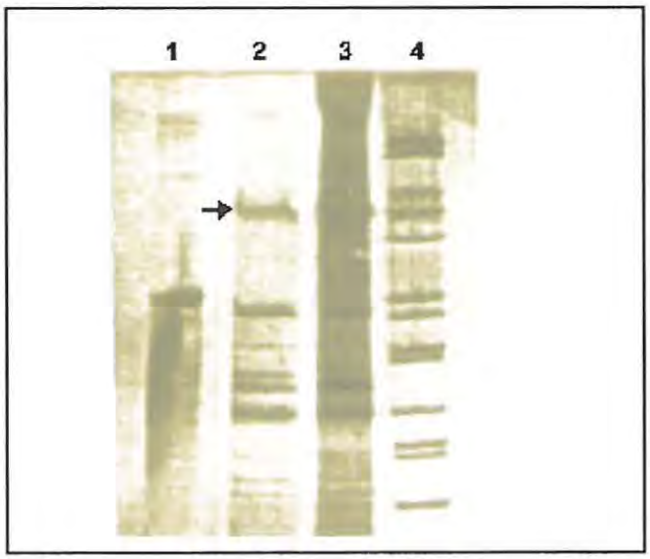

Figure 2.7 Amplification products obtained with an annealing temperature of 62°C maintained for 20 seconds, followed by 1 minutes extension and 30 seconds denaturation in an Idaho Technologies capillary thermal cycler. Re-designed *hrp* forward primer 5'CATGATCCTTTCGACCTACATCT3' and 21mer reverse primer 5'-CTGCAGTGCGCGCCCGGTCA-3' designed on the basis of *hrpXo* and *hrpXc* were used at concentraitons of 100μM each. 100μM of each dNTP, and 0.5U*Taq* were used in a 20μl reaction tube with 400ng template DNA. Lanes 2 and 3 contain amplification products obtained with 4mM and 2mM $MgCl_2$ respectively while lane 4 contains the molecular weight marker. The arrow indicates an amplification product of the correct size.

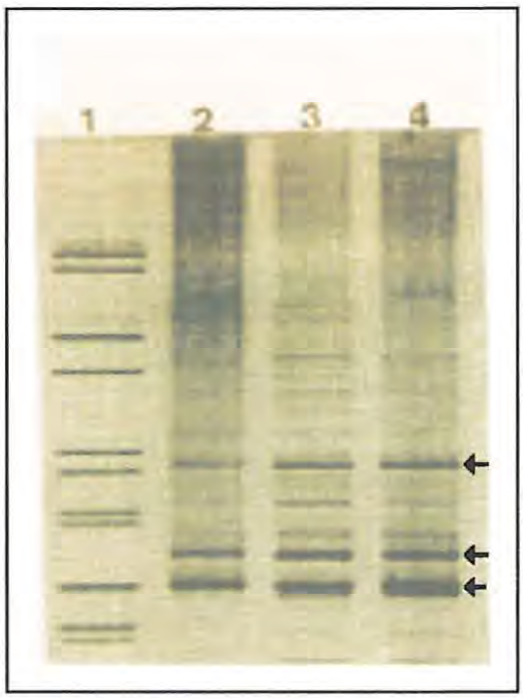

Figure 2.8 During the optimisation of annealing temperature using the newly designed forward primer, highly reproducible smaller amplification products were observed in the size ranges 300 to 600bp. The three brightest bands (indicated with arrows) corresponded to fragment sizes of 310, 360 and 580 base pairs.

No possible internal priming site was identified in known sequences at the annealing temperatures used, as identified by progressively lower stringency alignments between known sequences and primer sequences (See appendix IV). Despite the predominance of the smaller fragments, gel purification of a fragment of the expected size was performed and the resulting DNA subjected to restriction digestion. Restriction digests showed no similarity to predicted patterns based on *hrpXo* and *hrpXc* sequences.

2.4 Discussion and conclusion

A relatively high protease activity was observed on plate assays. The relative reduction of activity in the well assays could have been due to the half life of the enzyme in the wells during the long incubation period, which theory is supported by the apparent reduction in activity in concentrated fractions, possibly due to autocatalysis. The higher activities obtained in minimal media apparently contradict the findings on solid media, unless a cell density contribution is considered in the overall production and/or export of the enzymes. Since higher activities per cell were obtained at higher cell densities, a possible quorum sensing function could be contributing to the increased activity. This is further supported by the apparent lack of activity in liquid culture when assayed using the TCA protease assay method, which was probably due to both limited activity and a relatively short incubation period. In experiments where the incubation was extended for 24 hours, minimal activity was observed but this was considered insufficient for comparative studies. This then suggests a relatively low activity, a high degree of autocatalysis and the requirement for high cell densities. The effect of growth in minimal media on the increased export of proteins as assayed in wells suggests that the *hrp* genes play a major role in this regulation, since the expression of these genes is known to be enhanced on minimal media, and more evidence is becoming available to indicate the role of the *hrp* genes in protein export. This speculation is however tempered by the knowledge that pleiotropic enzyme-export deficient mutants can be generated (Denny *et al*, 1990; Hu *et al*, 1992) which maintain both their pathogenicity (albeit at reduced virulence) and ability to generate HR on suitable plants. It seems likely therefore, that more than on regulatory mechanism for the export of enzymes is present in the organisms where this has been shown to be the case.

For both the CMC and pectin, two distinct zones of substrate clearing were observed, with complete clearing occurring in the inner zone of the CMC plate, indicating complete

hydrolysis of the polymer. The actual identification of the enzymes was not the purpose of this screening and was therefore not pursued, since sufficient plant cell wall depolymerases were shown to exist, and sufficiently simple assays were shown to work, to allow the screening of vast numbers of mutants.

X. campestris pv. *zeae* was thus found to produce and export a wide range of potential plant cell wall depolymerizing enzymes, including at least one protease, cellulases including B-1,4-endoglucanase or carboxymethylcellulase, and either cellobiohydrolase or B-glucosidase, a possible pectic lyase (probably an endo-pectic lyase) and a polygalacturonase (although the presence of pectin methyl esterase cannot be excluded), in addition to the amylase. Unlike other Xanthomonads, *Pseudomonas spp.* and *Erwinia spp.*, no substrate induction for any of plant cell depolymerizing enzymes was observed under the assay conditions. Plate assays were not only relatively simple and rapid, but the results obtained were reproducible and the assays sensitive enough to detect multiple activities on one medium. Due to these good results, the plate assays for cellulase and endoglucanase, polygalacturonate and pectin lyase, amylase and protease, in addition to screening on solid media for variation in EPS production, auxotrophy and *in-planta* behaviour and virulence and pathogenicity, were used for screening of mutants (see chapter 3)

Hrp gene presence and activity was proven by the elicitation *in-planta,* of typical hypersensitive response on non-host plants and by dot blot analysis. The low similarity as determined by dot blot analysis, and the absence of bands in an optimised Southern blot experiment lead to the conclusion that there exists significant difference in the analogous sequences between probe and test DNA. This was as expected, since Arlat *et al* (1991) also report cross hybridisation only under low stringency conditions, although the extent of the difference as determined by the inability to get reproducible results even at low stringency's was not expected. On the basis of the variation in the restriction endonuclease sites in the 1220bp amplification fragment, and the consistent internal priming observed with the reverse primer, *X. campestris* pv. *zeae* was shown to have a significantly different *hrpX* gene to those of *X. campestris* pv. *campestris* and *X. campestris* pv. *oryzae.* Again, the purpose of this work was simply to establish the validity of attempting to generate and screen mutants (see chapter 3) for *hrp* gene functionality, and to provide a basis for analysis in the event of such mutants being isolated.

Having sufficiently shown the presence of several potential pathogenicity and virulence factors, and optimised screening procedures for these, it was possible to continue with the establishment of systems of mutagenesis and the subsequent generation, screening and analysis of mutants.

Chapter 3 - Mutagenesis and Mutant Screening

3.1 Introduction

In cases where there is no specific indication as to the nature of the molecular determinants of pathogenicity, or in cases where novel determinants are being sought, the identification and analysis of molecular determinants is most easily achieved if mutants deficient in the particular characteristic are isolated. By molecular and *in-planta* characteristic comparisons, it is often possible to identify the molecular basis for particular pathogenic or virulence features of the particular pathogen. Many examples of molecular determinants of various phenotypes, including bacterial plant pathogenesis determinants, have been elucidated using this approach (Shaw & Berg, 1979; Selvaraj & Iyer, 1983; Daniels, 1984; Turner *et al.* 1984; Thurn & Chaterjee, 1985; de Lorenzo *et al.* 1990; Herrero *et al.* 1990; Hötte *et al.* 1990; Bavage *et al.* 1991; Hu *et al.* 1992; Kolodrubetz & Kraig, 1994; Sharypova *et al.* 1994)

Although chemical mutagenesis is technically very simple, and generates many mutants in a short space of time, the nature of the mutations makes analysis difficult. Despite this, chemical mutagenesis has been used occasionally (Hwang *et al.* 1992; Daniels *et al.* 1984b) Complementation analysis (Kamdar *et al.* 1993; Gough *et al.* 1988; Hwang *et al.* 1992), using a suitable cloning vector, allows the isolation of the DNA fragment responsible for the lost phenotype, but only if the lesions are small or sufficiently close together to clone in one unit. Even allowing the use of cosmids this may pose a problem particularly since screening with mixed batches of complimented mutants is not ideal for *in-planta* work. The biggest problem with this approach, however, is the extent of mutagenesis required to make selection of mutants feasible, with multiple mutations being present at levels of mutagenesis that yield mutants at a viable frequency.

Transposons offer a method for generating tagged mutations which are easily recovered by simple cloning techniques. The transposons must insert randomly, and ideally at low copy number so as to facilitate unambiguous identification of the insertionally inactivated gene of interest. Transposon Tn5 has been used successfully to generate random insertion events in plant pathogens, and was thus selected as the transposon of choice. Tn5 was used by Thurn & Chaterjee (1985) to generate single-site insertions in the genome of *Erwinia chrysanthemi*.

Turner *et al.* (1984) failed to detect insertion of Tn5 ($<10^8$) into the genome of *X. campestris* pv. *campestris* from the delivery vector, while Tn7 was shown to insert in "hot spots" at orders of magnitude at least twice that of Tn5 when delivered from the pACYC184 replicons pSUP1011 and pSUP2011 using the RK2 *tra*$^+$ pRK2013 (Turner *et al.* 1984). Hu *et al.* (1992) however recovered Tn5 mutants of *X. campestris* pv. *campestris* using pSUP2021 at a frequency of 4×10^{-8}. Based on the lack of evidence for usefulness of other transposons and the wide range of mutagenic applications of Tn5 in diverse bacterial genera, Tn5 was considered the best option for mutageneiss of *X. campestris* pv. *zeae* despite the need for extensive testing and poor results reported by Turner *et al.* (1984) and Daniels (cited in Iyer, 1989).

Delivery of the transposon to the bacterial cell also presents a problem in that suitable narrow host range delivery vectors with appropriate transfer systems must be identified to shuttle the transposon into the recipient cell. Although some information is available for *X. campestris* pv *campestris* with regards mating frequencies and replicon stability's is available (Turner *et al*, 1984), there appears to be some variation between pathovars and no information was available on *X. campestris* pv *zeae*. *X. campestris* pv. *campestris* was shown to support P and Q group replicons but not pACYC184, ColE1 or pBR325. Kolodrubetz & Kraig (1994) used the pUT/mini-Tn5(Sp) vector (De Lorenzo *et al.* 1990), containing an RP4 *mob*, with SM10(λ*pir*) as the donor strain, to generate *Actinobacillus* mutants via conjugation that were found to be stable but were only recovered at a frequency of 10^{-7}. The pACYC184 based self mobilizable pGS9, containing the N transfer system from pCU1 (Selvaraj & Iyer, 1983), and it's *tra*$^-$ derivative, pGS39, transfered Tn5 to recipient *Rhizobium melilot* with less than 1% showing pACYC184 or pCU1 sequences when tested by Southern blotting. Selvaraj and Iyer (1983) reported Cm^R co-transfer in 3% of *Rhizobium meliloti* Tn5 mutants generated with pGS9 and 3% auxotrophs amongst the Cm^S exconjugants. Turner *et al.* (1984) reported a 3/1850 frequency of auxotrophs in *X. campestris* pv. *campestris* using Tn5 in pGS9, but no Km^R exconjugants when using pSUP2011 as the delivery vector despite success achieved with this delivery system or derivatives thereof in other organisms (Miller & Mekalanos,1988; Hötte *et al.* 1990) and despite the good transfer normally achieved using group P delivery systems. They did note, however, that Km^R was observed in exconjugants at a frequency $>10^{-3}$ when SM10 was used as donor strain in bi-parental matings, but with co-transfer of additional

markers. Based on literature, N group transfer systems appeared the most promising for transfer of transposons to *X. campestris* pv. *zeae*.

3.2 Methods and materials

3.2.1 Isolation of SAM116R and rifampicin resistant DH5α

SAM116R (a spontaneous rifampicin resistant mutant of SAM116) was isolated by selection on 80μg/ml rifampicin supplemented NA. 0.5ml aliquots of overnight culture of SAM116, grown in 200ml NB at 28°C and 180rpm were plated onto the selection medium and incubated at 28°C for 48 hours. Colonies were purified by re-streaking on the rifampicin-NA, and resulting single colonies were tested on a range of rifampicin concentrations in solid and liquid nutrient media. A single mutant strain capable of growing in the presence of 100 μg/ml in both liquid and solid medium was selected and stored in 20% glycerol at -80°C to serve as a source for this strain. This was done to prevent any attenuation that might occur due to multiple passage on artificial medium. The mutant was compared to the wild type isolate in all characteristics previously shown to be significant in pathogenisis, and for which suitable screening procedures had been developed. A spontaneous rifampicin resistant mutant of *E. coli* DH5α was isolated in the same way for use in selecting for newly constructed mobilizable narrow host range vectors.

3.2.2 Chemical mutagenesis and RAPD confirmation of Mutation

EMS was used as described by Miller (1972) to generate several hundred mutants which were screened with no result. Due to the cost and toxicity of EMS, mutagenesis using this chemical was discontinued in favor of nitrous acid. Nitrous acid was selected as the mutagen of choice due to it's relative low cost and toxicity, and the nature of the mutations generated. Mutagenesis was performed on a pellet obtained by brief centrifugation at 12,000g of 1.5ml early-log phase cells (OD 600nm of 0.3), washed 0.1M acetate buffer (pH 4.6), and resuspended in 600μl freshly prepared nitrous acid. Miller (1972) describes a large scale/volume protocol for nitrous acid mutagenesis using 0.5M sodium nitrite. Both this concentration, and 0.025M sodium nitrite, were used in 0.1M acetate buffer, in an attempt to yield optimal reproducibility. Incubation was at 37°C, and the reaction was halted by the addition of 1ml 0.1M phosphate buffer (pH7). Kill curves were constructed by sampling single mutation reactions at 30s intervals, and transferring the sample to the appropriate volume of

0.1M phosphate buffer at pH7, and by stopping reactions in different tubes at different times to establish reproducibility. Cells were pelleted and resuspended in 1ml LB medium and allowed the surviving cells allowed to double in number so as to minimize the percentage of sectored colonies and mixed mutant cultures. Conditions were optimized to yield reproducible production of mutants at 1% survival, 0.5% survival and 0.1% survival. Mutants were plated onto NA for later picking and screening, and onto those media which were suitable for direct screening.

RAPD analysis (Caetano-Anollés *et* al. 1991) was performed on selected mutants. Three 10mer primers were used, 5'-TCACATGGCA-3', 5'-TCGATGAACG-3' and 5'-AACCGATGCT-3'. DNA isolations were performed as described by Sambrook *et al.* (1992). 100ng total DNA was used in each reaction tube which contained 400µM dNTP mixture, 100µM of the appropriate 10mer primer, 1,5mM $MgCl_2$ and 1U*Taq* DNA polymerase. 40 cycles of 37°C, 72°C, 94°C for 20s, 60s, and 30s respectively were employed after an initial five minute denaturation at 94°C, and a final extension at 72°C for three minutes was used to complete the reaction Analysis of PCR products was on a discontinuous 10% SDS PAGE gel, but using 1xTBE as the running buffer. Gels were silver stained with a modified protein silver staining procedure which consisted of 0.12M silver nitrate for 15 minutes after an initial fixing in 10% (v/v) ethanol plus 5% (v/v) glacial acetic acid for 15 minutes, followed by a reduction step in 0.0034M potassium dichromate with 0.0032N nitric acid for 10 minutes, which was then completely washed out with several rinses of de-ionized water. Development was in 0.28M sodium carbonate containing 0.15% (v/v) formaldehyde. Upon the appearance of bands at the desired intensity, as monitored by molecular weight markers of known concentration, the reaction was stopped with the fixative.

3.2.3 Transposon mutagenesis

Transposons were used in an attempt to produce tagged mutations so as to facilitate the recovery of insertionally inactivated genes. It was however first necessary to determine which origins of replication would be supported by the *X campestris* pv. *zeae* and which would not, which mating system would prove to be the most efficient in the transfer of shuttle vectors and suicide vectors between *E. coli* and *X. campestris* pv. *zeae*, and which transposons would

yield relatively high rates of random insertion events with the lowest number of multiple insertion events so as to facilitate easier recovery of tagged genes.

3.2.3.1 Screening of origins of replication and transfer

Suitable vectors were required for the maintenance of cloned material in *X. campestris* pv. *zeae*, and to act as suicide vectors for transposon mutagenesis and site specific insertional inactivation by homologous recombination with cloned gene fragments suspected of being involved in pathogenicity. SAM116R was tested for its ability to maintain various plasmid vectors received from *E. coli* donors by conjugation.

All cultures were grown to mid log phase, and matings were performed on 0.45μm filters by mixing appropriate amounts of the cultures to yield a 1:1 ratio of donor to recipient, or in the case of tri-parental matings, a 1:1:1 ratio of donor, helper and recipient, followed by pressure filtration via a 10ml syringe. Filters were incubated bacteria upward on nutrient agar plates at 30°C for sixteen hours. Filters were placed in 3ml sterile saline and the bacteria resuspended by vortexing. Serial dilutions were plated onto the appropriate media to select for exconjugants and recipients, and the mating efficiency was calculated as number of exconjugants per recipient present on the filter membrane after the sixteen hour incubation period. Antibiotic concentrations used (in μg/ml) were as follows:Rifampicin 80, Kanamycin 10-50, tetracycline 5, Chloramphenicol 15-50. Lower ranges of antibiotic concentrations were for selection of low frequency insertions or stringent plasmids. Alkaline lysis mini-preps were performed as described by Sambrook *et al* (1992) to test qualitatively and semi-quantitatively, by comparison of relative band fluorescence intensity on agarose gels, for levels of maintenance.

RP4 and RK2 replicons were initially tested to confirm the functionality of the transfer systems, whereafter, in the absence of a self mobilizable vector, SM10λpir, an RP4 Cb^s Tc::Mu, was used to mobilize unless a specific helper plasmid was available. In all cases where the chromosomally integrated transfer functions were used, mobilization was confirmed with the isolated rifampicin resistant DH5α. The following vectors were tested for maintenance in SAM116R:

♦PR6K *ori* based, pGP704 (*mob*$^+$-*RP4*, Ap^R) (Miller & Mekalanos, 1988)

♦the ColE1 replicon pRK2013 (*Tra*$^+$, *RK2-mob*$^+$, Km^R) (Figurski & Helinski (1979),(Ditta *et al* (1980)),

♦the IncW pSA747 (*mob*$^+$, Km^R, Sp^R, *cos*$^+$) (Tait *et al*, 1983)

♦the RFS1010 based pDSK519 (Km^R, *lacZ*) (Keen *et al*, 1988)

♦the IncW, pSA *ori* containing pUFR027 (Km^R, *lacZ, parA*) (De Freyter *et al*, 1990)

♦pUFR047 (as for pUFR027 but Gm^R, Ap^R) (De Freyter & Gabriel, 1991)

♦the P group cosmid pLAFR1 (Tc^R, *tra*$^-$, *mob*$^+$, RK2 replicon) (Friedman *et al*, 1982)

Selection in all cases was by appropriate antibiotic resistance, and confirmation by mini-prep plasmid isolation and electrophoresis. Where necessary, restriction digests were used to confirm plasmid identity.

3.2.3.2 Screening of transposons

Transposon delivery vectors were tested as described in 3.2.3.1 for delivery to the host. Insertion was confirmed by growth on the appropriate selective medium and the absence of the delivery plasmid in the recipient. Tn5 sequences in chromosomal DNA were confirmed by dot blot analysis using the same conditions as for the Southern blot described below. Number of insertion events and randomness of insertion was determined by Southern blotting. Transposon recipient chromosomal DNA was isolated as previously described and digested, as per manufacturers specifications, with *BamHI* obtained from Boehringer Mannheim. *BamHI* was selected as it yielded a single cut in the inserted transposon, and thus afforded the opportunity to establish the randomness of insertion by determination of variation in sizes of the two fragments generated by digestion. The resultant digests were electrophoresed in 0.8% Seakem agarose with TBE at a constant voltage of 2V/cm for 20 hours. The resultant gel was blotted onto Hybond+® positively charged nylon membrane by capillary action using 0.4M NaOH as the transfer and denaturing buffer. DNA was fixed to the membrane by baking at 100°C for 30 minutes. Pre-hybridization, hybridization and detection were done using the DIG blocking, random labeling and colourometric detection system as per manufacturers specification, in a Hybaid Micro-4 hybridazation oven at 68°C for standard hybridization

buffer, or at 42°C wher DIG Easy-Hyb was used. Prehybridizations were carried out for a minimum of 3 hours and all hybridizations were overnight. Washes were performed in the hybridization oven bottles at high stringeny (68°C) unless otherwise specified.

The following Tn5 transposon-vector systems were tested in matings with SAM116R:

- pSUP2021 - *Tra*$^-$, *mob*$^+$*(RP4)*, Cm^R, Cb^R, Tn5, pBR325 *ori(E1)* (Simon *et al*, 1983)
- pGS9 - *N-Tra*$^+$, *mob*$^+$, Ap^R, Cm^R, Tn5, pACYC184 *ori* (Selvaraj & Iyer, 1983)
- pGS39 - *N-Tra*$^-$, *mob*$^+$, Ap^R, Cm^R, Tn5-*ori*T, pACYC184 *ori* (Hengen & Iyer, 1992)
- mini-Tn5-Tc - *Tra*$^-$, *mob*$^+$*(RP4)*, Ap^R, Tn5 Km^S, Tc^R, R6K *ori* (De Lorenzo *et al*, 1990)

3.2.3.3 Optimization of mating conditions

Once a suitably efficient delivery system was identified, and a transposon selected, the combination was optimized for highest yield of low insertion number/transconjugant insertions. Optimization parameters included ratio of donor:recipient or, in the case of tri-parental matings, donor:recipient:helper, duration of mating filter incubation, cell density and stage of growth prior to mating. Matings were performed as before on 0.45μm filters and incubated on nutrient agar prior to resuspension in sterile saline for selection and enumeration.

3.2.4 Vectors for mutagenesis by homologous recombination

For selection and construction of suicide vectors for insertional modification by homologues recombination plasmid preparations were done using QIAGEN spin columns as per manufacturers specifications, and chromosomal DNA isolations were from overnight cultures in selective media as described by Troyer *et al* (1990). DNA manipulations were as described by Sambrook *et al* (1992). Two narrow host range plasmids were constructed. pIN101 contained an RP4 *mob* derived from pUFR027 (De Freyter & Gabriel, 1991) and pRUT320 contained an RK2 derived *oriT*. These vectors were designed for ease of cloning, for their ability to mobilize into a spontaneously generated rifampicin resistant DH5α using a previously identified transfer function which yielded high levels of exconjugants in SAM116R, and their inability to be maintained in *X. campestris* pv. *zeae*. Figures 3.1 and 3.2 show the construction of the two narrow host range vectors from pUFR027 and pUC18 (Boehringer Mannheim) and pNH-Kan/*oriT* (Hengen & Iyer, 1992) and pBR322 (Boehringer Mannheim), and figure 3.3 shows the vector pGP704 (Miller & Mekalanos, 1988), obtained after the construction of the two suicide cloning vectors, which is equally suitable despite the lack of good cloning sites and selection. In all cases, enzymes used were obtained from (Boehringer Mannheim) and

digestions were performed as per manufacturers specifications. The kan/oriT cassette was purified from a 1% low melting point agarose gel (SeaKem Nusieve) after separation using TAE as the buffer, by excising the desired band, breaking up the agarose and placing the homogenate into 0.5ml microfuge tubes with a small hole in the base blocked with acid

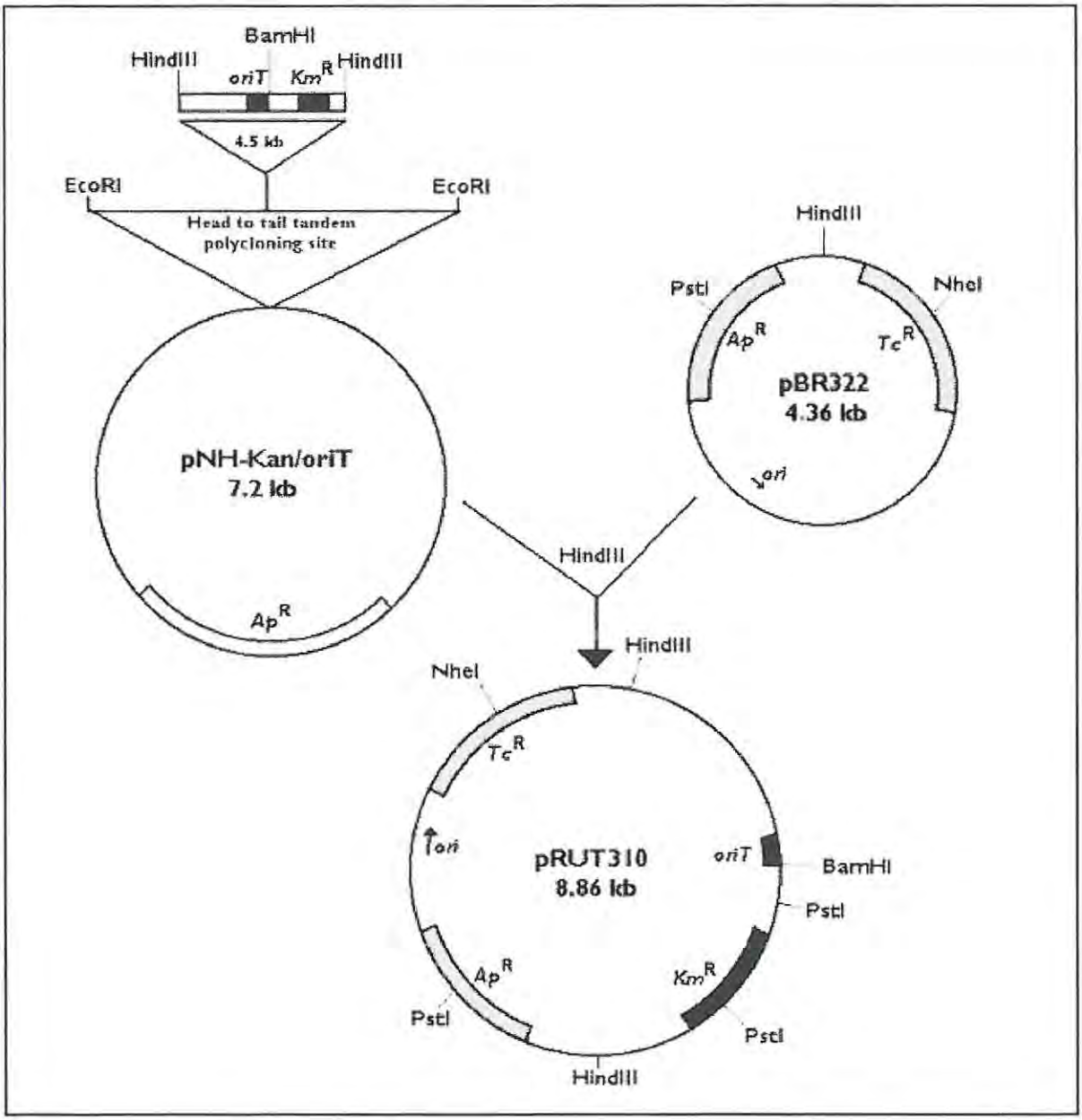

Figure 3.1 The simple construction of the narrow host range, mobilizable vector, pRUT310, with suitable markers and unique cloning sites. pNH-Kan/*ori*T (Hengen & Iyer, 1992)

washed glass wool. After snap freezing in liquid nitrogen, centrifugation at 12,000g resulted in elution of the DNA fragment in buffer into the 1.5ml microfuge tube in which the smaller tube was placed prior to centrifugation. The DNA fragment was then precipitated using isopropanol and washed with ethanol using standard methods, prior to resuspension in TE buffer (pH

7.5). All ligations were performed as described by Sambrook *et al* (1992) using T4 ligase obtained from (Boehringer Mannheim).

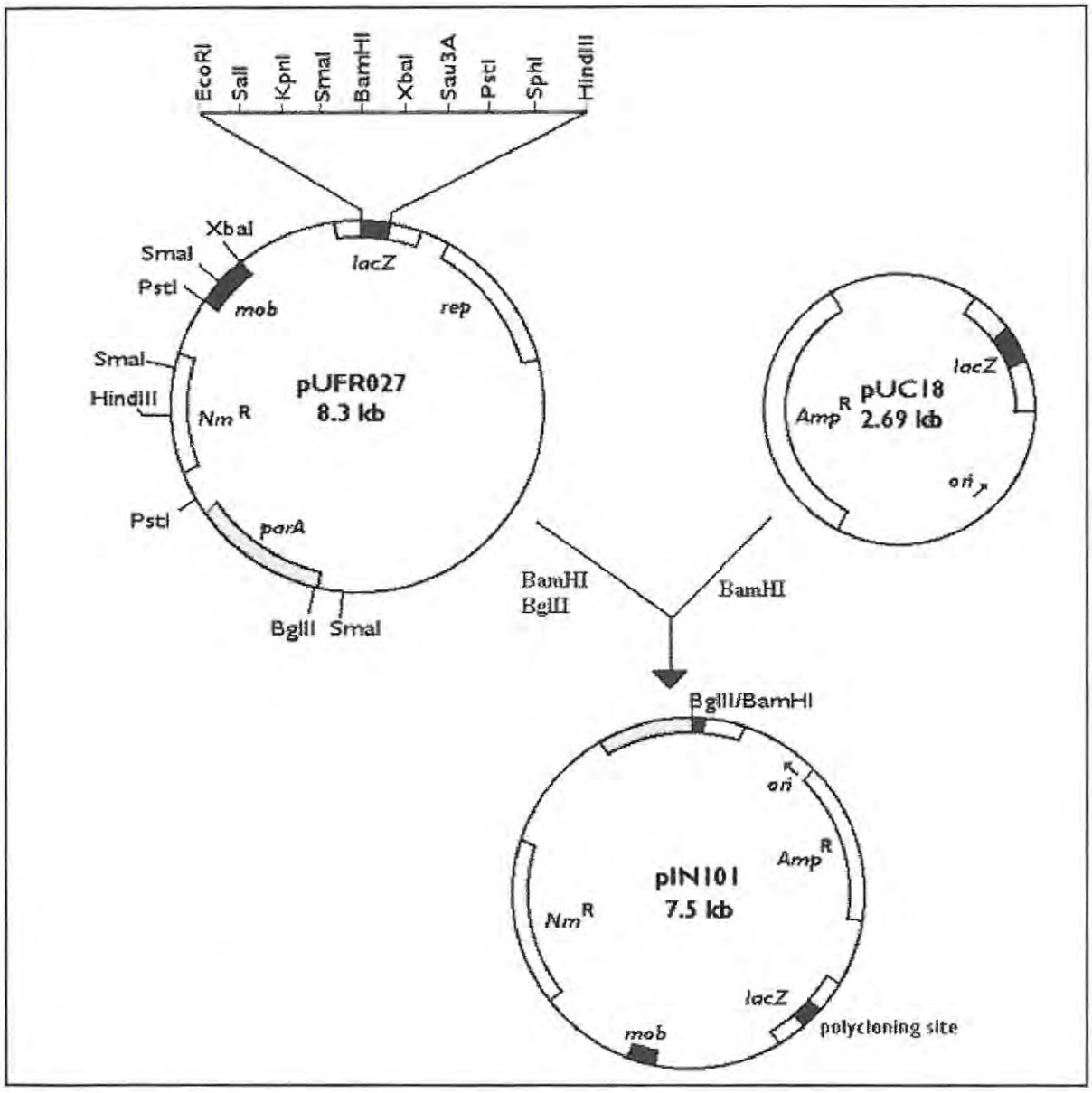

Figure 3.2 Construction of pIN101 from pUFR027 and pUC18/19 so as to maintain a functional *lacZ* gene.

3.2.5 Inoculation strategies

Various inoculation strategies were used in order to find a strategy which yielded rapid lesion development with minimal initial tissue damage, and most reproducible lesion development with a given innoculum. Bacterial suspensions were inoculated at various concentrations, into ten replicates of host and non-host (for HR purposes) using pressure infiltration of a fixed volume of bacterial suspension into the leaf, by injection of a small volume into the central

vascular bundle of the largest leaf, by pipetting a fixed volume of various concentrations into the central whorl of the seedling and either puncturing the whorl on both sides using a 21G needle, or leaving the seedling intact, or by using a double needle (sewing machine needle, Singer 12) dipped into the appropriate concentration of bacterial suspension and then used to puncture the leaf adjacent to the central vascular bundle. CFU/ml on the double needle were also measured by resuspension, dilution and plating onto appropriate medium to determine the reproducability of this method. In addition to these methods, inoculation from pure culture colonies by means of a sharpened wire (0.8mm diameter) which could be heat sterilized, was also tested in terms of reproducibility

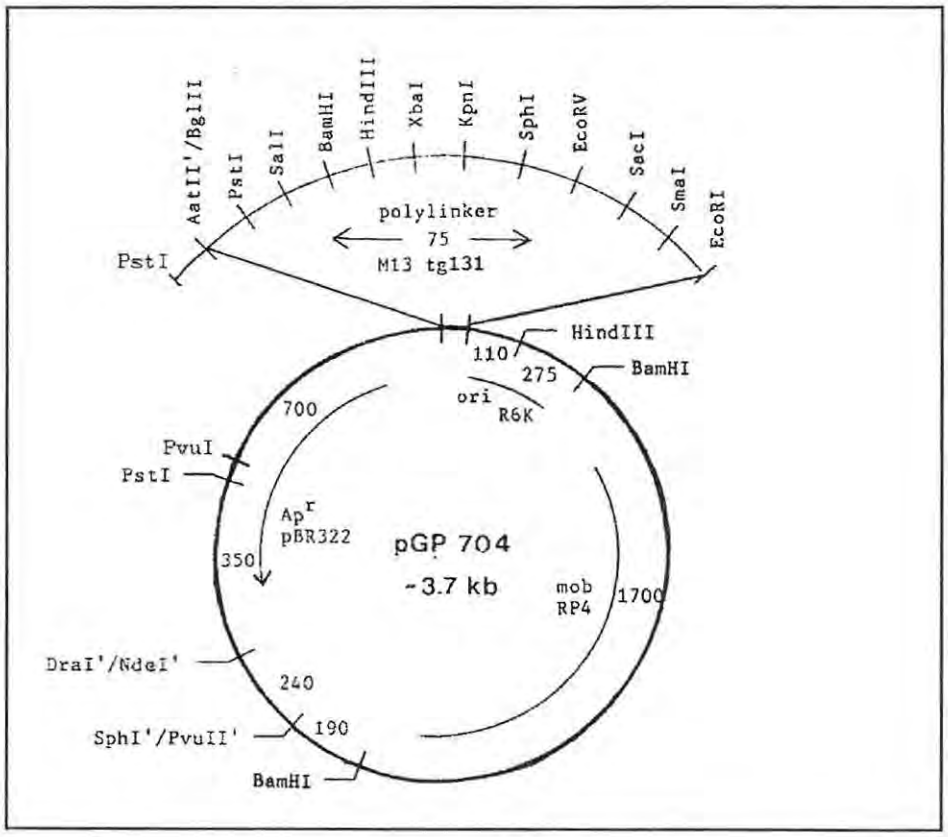

Figure 3.3 The narrow host range mobilizable vector pGP704 (Miller & Mekalanos, 1988)

3.2.5.1 Maize lines

Two maize lines were tested for their susceptibility to *X. campestris* pv. *zeae*. Pannar seed, lines I137Tn and U267Y, were used as reported susceptible and resistant lines respectively.

3.2.6 Generation and Screening of mutants

Mutants were generated using nitrous acid and Tn5. For Tn5 mutagenesis the pGS9 delivery system (See figure 3.4) was used and incubation for mating was allowed to proceed for only eight hours so as to reduce the possibility of screening clones.

All nitrous acid mutagenized bacterial isolates and Km^R transconjugants were screened by isolating pure culture of the mutant, picking onto CMC agar, pectin agar, purple milk agar, M9 minimal medium, sucrose supplemented nutrient agar and starch agar. In addition to screening for altered extracellular enzyme production or export, each mutant was needle inoculated into week-old maize seedlings, and pressure inoculated into tobacco leaves as previously described, to detect any alteration in pathogenicity or virulence, and to determine the mutant's ability to elicit HR, respectively. Each inoculated maize seedling was monitored for onset and rate of lesion development, and wilting. In each case, one in each ten test organisms (one colony per plate, or one in ten seedlings or leaf inoculums in the case of the tobacco plants) was the strain SAM116R, while one in every 25 plant maize inoculums was sterile water and one sector per tobacco leaf was inoculated with sterile water as controls.

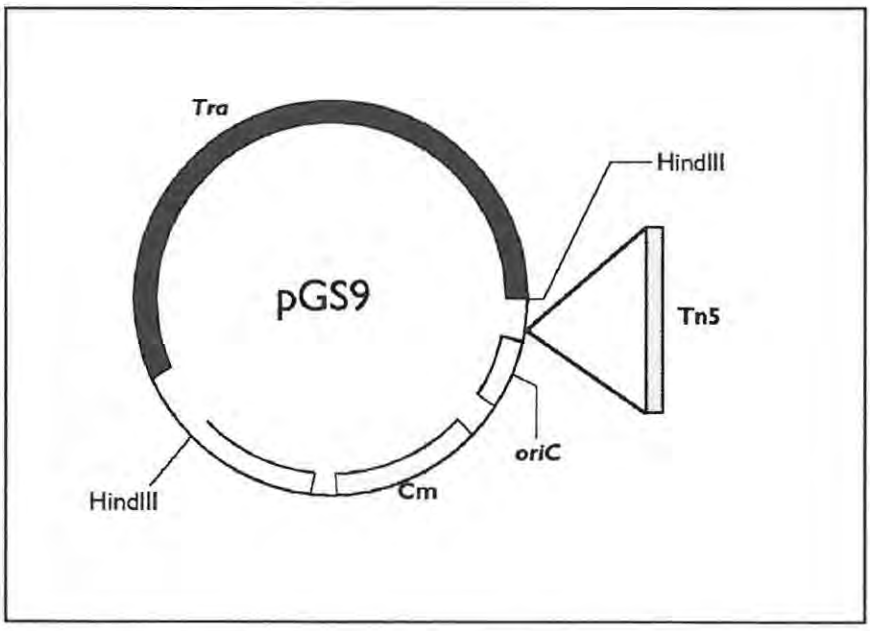

Figure 3.4 pGS9 (Selvaraj & Iyer, 1983) was used for generation of all Tn5 mutants

3.2.7 Confirmation of Mutant Phenotype

Where any mutants showed any alteration in any of the characteristics screened, or any deviation from the wild type, the mutant in question was subjected to re-testing on plates with

the wild-type, or on five plants in the same seedling tray as five seedlings inoculated using the double needle method, with SAM116R. Where plate assay's indicated a reduction in a particular plant cell wall degrading enzyme, crude enzyme extracts were subjected to well diffusion assays as previously described.

3.3 Results

3.3.1 Isolation of SAM116R and rifampicin resistant DH5α

SAM116R was isolated as described and compared with the wild type in terms of extracellular enzyme activity, growth rate on solid and in liquid medium, ability to elicit HR on tobacco, tomato and green pepper, colony morphology and appearance, growth rate *in-planta* and pathogenicity. In all cases there was no significant difference between SAM116 and SAM116R (see appendix V). A rifampicin resistant DH5α was also isolated as described.

3.3.2 Chemical mutagenesis and RAPD confirmation of Mutation

No significant differences were observed in the two methods used to obtain kill curves, indicating the high level of reproducibility obtainable despite the small scale reaction. Figure 3.5 shows the kill curve obtained from 3 sets of 10 samples from the same reaction tube, and 3

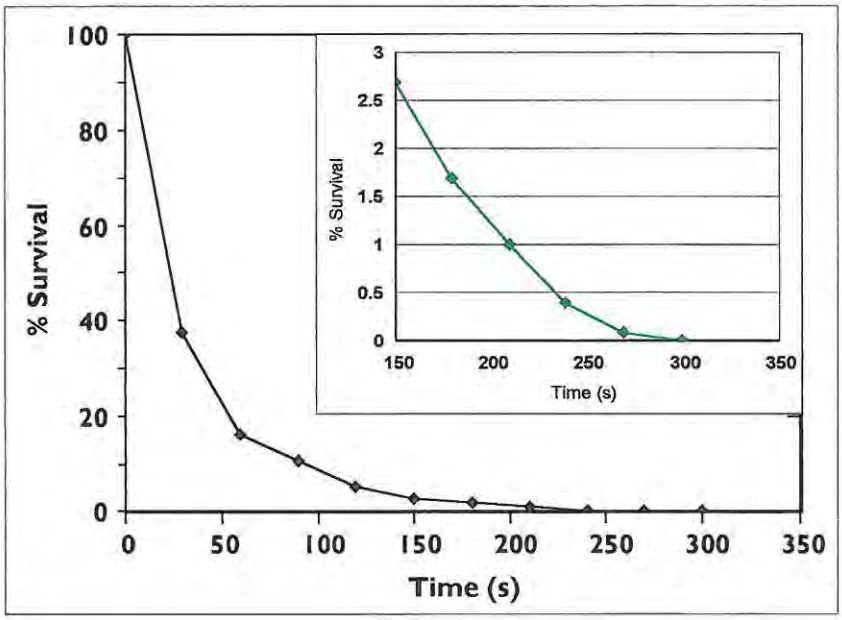

Figure 3.5 Survival curve of SAM116R exposed to nitrous acid. All points are averages of 5 replicates. Insert shows survival rates between 150 and 300 seconds. The linear scale shows more clearly the absence of a shoulder characteristic of ineducable repair.

sets of data derived from multiples reaction tubes. No significant variation was observed.Deviations from the mean were negligible (<0.1%) at times exceeding 3 minutes. Initially, mutagenesis was carried out at 180 seconds giving 1.5% survival. Mutants showing altered pathogenicity features were isolated at a frequency of $< 2.5 \times 10^{-4}$ (a single mutant showing significantly altered pathogenicity in 4011 screened in maize seedlings) while a single mutant showing reduced protease activity (40% reduction as measured by radial diffusion assay) was obtained from a total of over 7000 (a frequency of $< 2 \times 10^{-4}$).

Several mutants were compared to SAM116R using RAPD fingerprinting, to establish that mutagenesis was succesful, and that the extent of the mutagenesis was not insufficient to yield acceptable levels of useful mutants. RAPD analysis revealed a high level of mutation even at 1.5% survival. Typically, a 10mer yielded between 15 and 30 bands, and using the 3 primers, the number of bands different from SAM116R: total number of bands, were 2:23, 1:30, and 2:27. Figure (3.6) shows typical results obtained when comparing mutant and SAM116R profiles. No variation was observed between SAM116 and SAM116R.

Considering the small fraction of the genome sampled by RAPD fingerprinting, the extent of mutagenesis was considered to be relatively high. However, due to the low frequency of mutants showing altered pathogenicity, and the lack of mutants showing altered enzyme production in the first 2250 mutants screened, all further mutants were selected at 240s or 0.5% survival, including over 2000 mutants screened on protease and pectinase assay plates.

3.3.3 Transposon Mutagenesis

3.3.3.1 Screening of origins of replication and transfer

Matings of HB101/RP4 X SAM116R under test conditions produced a surprisingly high yield of Km^R exconjugants at a frequency of 0.4/recipient under excess donor, and a frequency of 0.23/recipient at a 1:1 donor:recipient ratio. Tc^R exconjugants were recovered at a frequency of 2.45×10^{-2}/recipient from tri-parental matings of ED8767/pRK2013 X HB101/pLAFR1 X SAM116R compared to 3×10^{-4} reported by Turner *et al* (1984) for *X . campestris* pv *campestris*. Km^R exconjugants were recovered at a frequency of 8.6×10^{-3}/recipient from tri-parental matings of DH5α/pDSK519 using pRK2013 as the helper plasmid in ED8767.

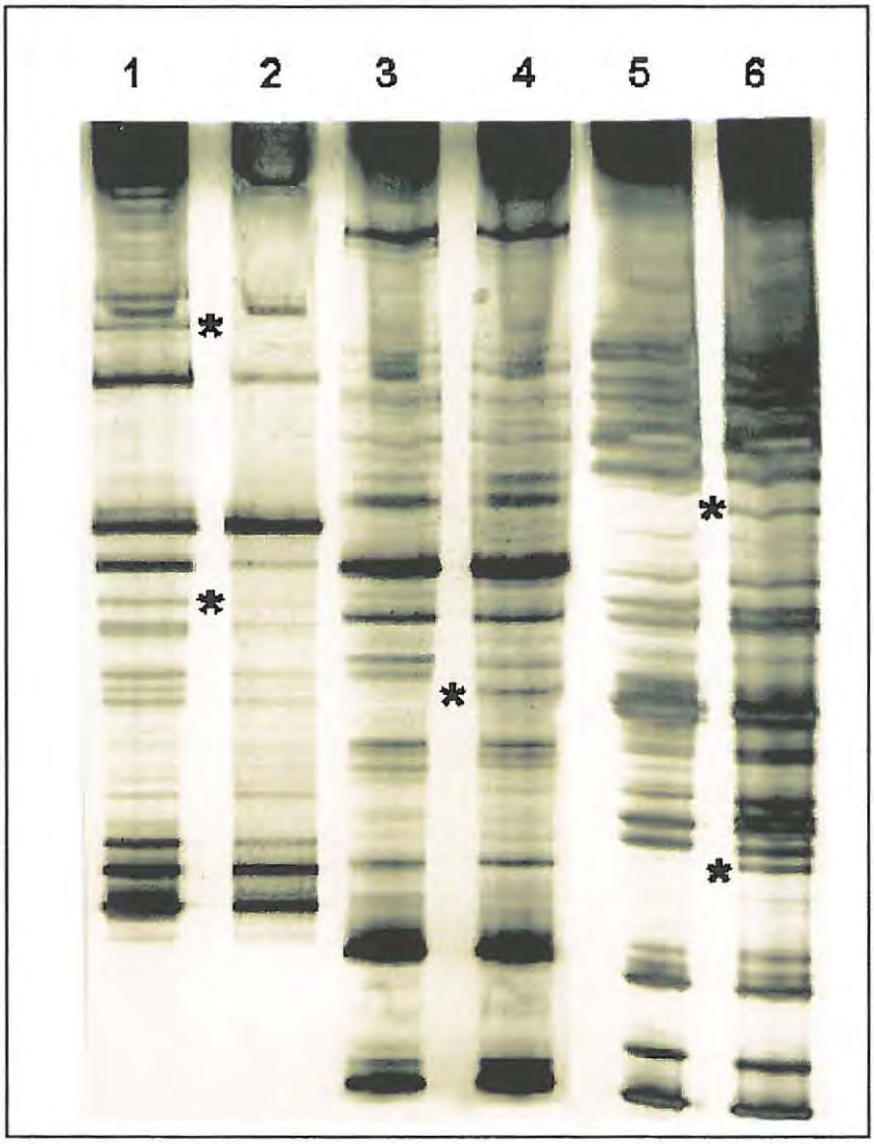

Figure 3.6 Typical RAPD fingerprints obtained when comparing SAM116R with nitrous acid mutants. Asterisks (*) indicate differences in profiles generated from SAM116R and A204 using the three primers described in the text.

Using the same helper strain in tri-parental matings with SAM116R and DH5α/pUFR027 or TB1/pUFR047, exconjugant frequencies/recipient were 4×10^{-2} and 1.9×10^{-2} resepectively. pSA747 failed to yield any transconjugants ($<10^{-9}$/recipient) in tri-parental matings with HB101/pSA325 as the helper strain. Failure to recover pSA747 from a mating with a rifampicin resistant *E. coli* as recipient indicated a faulty helper plasmid rather than failure to be maintained by *X. campestris* pv. *zeae*. In Matings between SAM116R and either HB101/pRK2013 or HB101/pSA325, no transconjugants were recovered ($<10^{-9}$).

3.3.3.2 Screening of transposons

pSUP2021, pGS39 and mini-Tn5-Tc, failed to yield Km^R or Tc^R SAM116R in mating experiments. Mini-Tn5 should have yielded good transfer efficiencies as the RP4 *mob* was shown to be highly efficient in *X. campestris* pv. *zeae*. Low Tc^R exconjugant recovery should be attributed to poor insertion frequencies as a result of the manipulation to the transposon (De Lorenzo *et al*, 1990). Kolodrubetz & Kraig (1994) obtained poor yields of exconjugants, despite good transfer efficiencies using the RP4 *mob*. pSUP2021 was reported not to yield Km^R exconjugants in tri-parental matings with various *X. campestris* pathovars (Turner *et al*, 1984). Using SM10 as a donor strain, however, yielded Km^R exconjugants at a frequency $>10^{-3}$/recipient but with co-transfer of integrated RP4 resistance genes. No exconjugants were recovered carrying any markers from any of the above transposons, when using either tri-parental matings or using either SM10 or S17.1 as the donor strain. HB101/pGS9 x SAM116R yielded the highest frequency of exconjugants, 9.6×10^{-3}, making this the Tn5 delivery system of choice. Unlike the relatively high yield of auxotrophs obtained with Tn5 by Turner *et al* (1994) on *X. campestris* pv. *campestris* and by Selvaraj and Iyer (1983) on *R. meliloti*, Tn5 delivered with pGS9 failed to yield auxotrophs in 200 initial exconjugants tested. At 15µg/ml Cm (Turner *et al*, 1984), 100% Cm^R was observed in Km^R SAM116R exconjugants. At a concentration of 50 µg/ml (Selvaraj & Iyer, 1983), only 0.75% of exconjugants showed co transfer of Cm^R. Dot blot analysis (data not shown) using the Tn5 fragment excised by gel purification from pGS39 (Hengen & Iyer, 1992) as the probe, showed 100% presence of Tn5. Southern blot analysis showed random insertion of the transposon with frequent multiple insertions (See figure 3.7).

Results for other blots performed as described, yielded predominantly single site insertions with 83% (10/12) of isolates showing only two bands on the blot. A total of 12/14 or 85.7% of insertions were thus single site insertions, although two (lane 5 figure 3.7) and even three (lane 4 figure 3.7) insertions were not uncommon.

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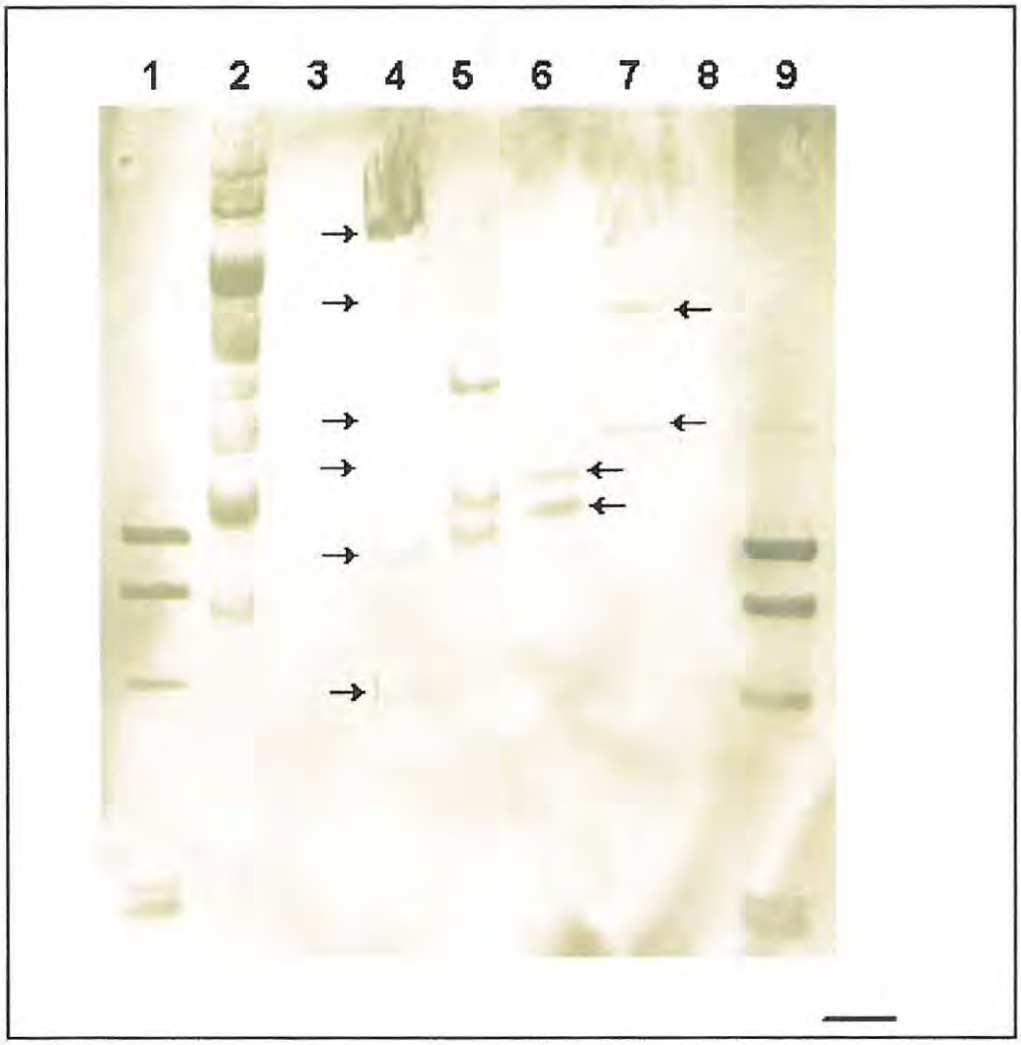

Figure 3.7 Southern blot of *BamHI* digested total DNA after separation on 0.8% agarose in TBE for 20 hours at constant voltage of 2V/cm, performed using DIG labelled pGS39 as a probe at 68°C followed by high stringeny washes prior to detection with anti-DIG alkaline phosphatase conjugate using NBT and X-phosphate as substrate. Lanes 1 and nine contain Boehringer Mannheim molecular weight marker II. Lane 2 contains a *HindIII* digest of PGS9. SAM116R (Lane 3) and HB101 (Lane 8) have no visible bands . Lanes 4 to 7 are SAM116R::Tn5 digests showing single and multiple insertions. Arrows indicate the position of bands in lanes 4, 6 and 7, while the three bands in lane 5 are clearly visible.

3.3.3.3 Optimization of Mating Conditions for HB101/pGS9 and SAM116R

Figure (3.8) shows the effect of donor:recipient ratio's on the efficiency of mating and Tn5 transposition using HB101/pGS9 as the donor/vector combination. A clear trend showing the exponential relationship between donor:recipient ratio and transfer efficiency was observed. Highest transfer efficiencies (0.015) were obtained with donor/recipient ratios of 3.3×10^{5}. A 1:1 ratio yielded exconjugants at approximately 2×10^{-5}, while at ratios below 0.01, efficiencies below 10^{-9} were typical.

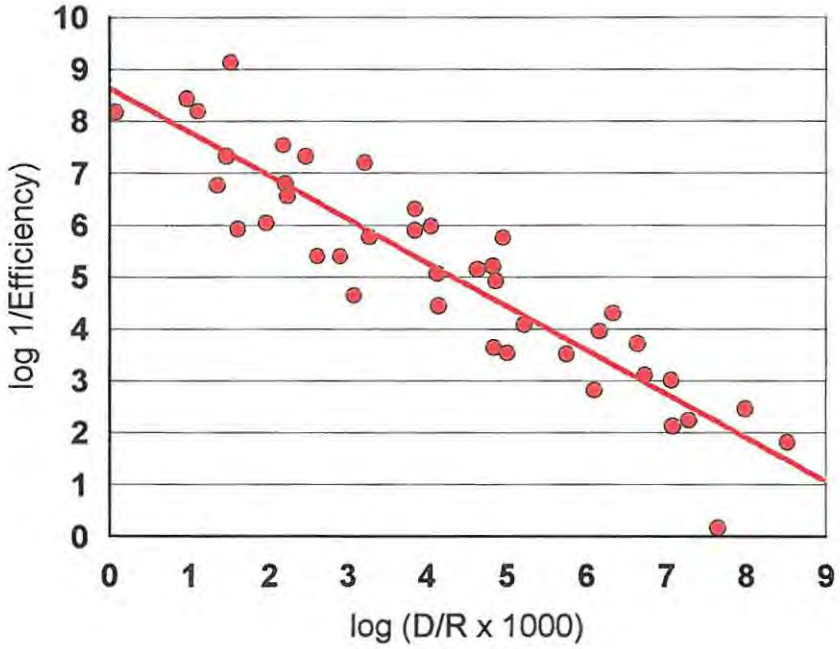

Figure 3.8 Transfer efficiency as a function of cell donor:recipient ratio yielded an R-square of 0.836 upon regression analysis. A descending scale was used on the abscissa as the data was an inverse figure.

Deviation from the linear regression was measured and plotted against cell density derived from numerous experiments, in an attempt to explain the relatively low R-square (See figure 3.9).

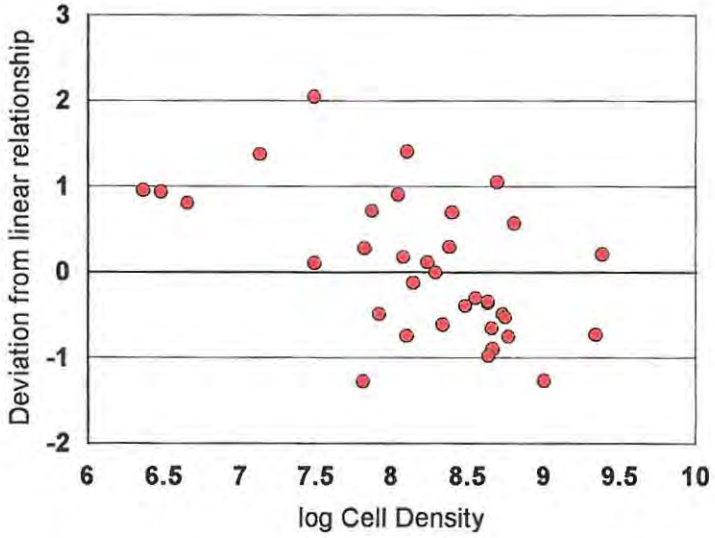

Figure 3.9 Deviation from the regression analysis obtained from the relationship between donor:recipient cell ratio and transfer efficiency. The deviation was plotted against the absolute cell density on each individual filter.

Although not immediately apparent, a trend was observed with reduction in relative efficiency at high cell densities. Duration of mating was found not to effect the efficiency in any significant way other than that a minimum time of 6 hours was required to obtain reproducible results. Longer times, yielding higher cell densities also resulted in higher efficiencies but presumably as a function of cell density since no appreciable increase was found at comparable cell densities with varying incubation times. In fact, incubation times were generally kept below 8 hours so as to limit the possibility of reproduction of exconjugant::Tn5 organisms and cause unnecessary screening of mutant clones.

3.3.5 Vectors for mutagenesis by homologous recombination

In mating experiments using SM10λ*pir* as the donor for pIN, pRUT320 and pGP704, native plasmids were not maintained in recipient SAM116R cells. No integration was observed with any of the plasmids used. SM10 was able to mobilize the pRUT320 and pGP704 into DH5αR at high frequencies (>0.1/recipient) where it was maintained and from where it was isolated to confirm the transfer, but failed to mobilize pIN101. When HB101/pRK2013 was used as a helper to mobilize pIN101 from HB101 into DH5αR on the assumption that the *tra* and *mob* functions of RP4 and RK2 may not result in efficient transfer, still no Ap^R DH5αR was obtained ($<1 \times 10^{-10}$). When appropriately digested fragments of SAM116R total DNA were cloned into each of the plasmids using standard techniques (Sambrook *et al*, 1992), mating efficiencies to DH5α were unaltered. pRUT321 and pGP704f (being recombinant pRUT320 and pGP704) containing 465bp and 240bp fragments of SAM116R DNA respectively, yielded exconjugant::pRUT321 and exconjugant::pGP704 at average frequencies of 4.2×10^{-9} and 8.3×10^{-9} per recipient respectively as an average of three experiments. Site specificity and recombination rates were not determined because of the lack of unique sites to facilitate easy cloning of fragments for site specific insertional inactivation by homologous recombination, and because of the relatively high cost of generating a single mutant and lack of true randomness in cloning because of the limited cloning sites. The system was however suitable for mutagenesis with previously cloned genes, and results obtained indicated the potential value of such a system for site specific work once genes implicated in pathogenicity or virulence have been cloned.

3.3.6 Screening of isolated bacteria

5231 Tn5 containing exconjugants and 4011 nitrous acid mutagenized bacterial isolates were purified and screened for any alteration in the production or export of any of the enzymes discussed, and for any alteration in pathogenicity or virulence, or in the ability to elicit HR on tobacco plants. In addition to these, over 3000 mutants were screened directly for alterations in protease and pectinase production. Direct screening for pectinase production was done by lifting colonies on sterile filter paper disks, prior to staining with congo red as previously described, so as to facilitate recovery of any potential pectinase mutants.

3.3.6.1 Enzyme production and export, colony and growth characteristics of isolates

A single mutant generated by nitrous acid mutagenesis, and recovered at a survival rate of 0.5% was isolated and designated A3345. The mutant showed consistently decreased protease production when cultured on milk medium, and yielded reproducible smaller zones of clearance in well diffusion assays. No obvious variation in *in-planta* behavior was observed during screening or mutant confirmation tests on A3345. No other phenotypically distinct colonies were observed. The low frequency of auxotroph isolation, prompts questions as to the lethal nature of nutritional mutations in *X. campestris* pv. *zeae*. Although chemically induced mutants could conceivably contain multiple mutations, reducing the probability of obtaining the desired mutant at the survival rate used, transposon mutants do not present this problem, and the failure to isolate auxotrophs ($<1.88\text{x}10^{-4}$) is highly anomalous and cannot be explained since mutagenesis and purification prior to selection were on complex rich medium.

3.3.6.2 Mutant *in planta* behavior

Needle inoculation with size 12 sewing machine needle at a position half way up the leaf from the stem and half way between the central vascular bundle and the leaf margin gave the most reproducible results. Inoculum concentration was found to have a limited effect on results, unlike results obtained by Kao *et al* (1992) with *P. solanacearum*, but growth phase of the culture was found to be significant in the pathology. Inoculation from colonies using a sharpened wire was surprisingly reproducible, probably due to the low significance of inoculum concentration in the pathology.

A total of 23 nitrous acid mutants and 209 Tn5 induced mutants showed deviant behavior in *in-planta* assays. These were re-tested as described to confirm screening results. In almost all

cases, these variations were not found to be reproducible. Where results were reproducible, mutant strains were immediately stored as previously described and subjected to analysis. The nine validated mutants with altered *in-planta* behavior are described below. Name designation was based on method of mutagenesis, batch and plate isolate number.

A single non-pathogenic mutant (A204) was isolated at 1% survival after chemical mutagenesis using nitrous acid. *In-planta* results were confirmed on five seedlings. A single putative *hrp*⁻ Tn5 induced mutant was isolated at a frequency of 1.5×10^{-4} and was designated SB41026. SB41026 failed to yield HR on tobacco and caused no symptom development in maize seedlings, but had wild type colony and growth characteristics. Tn5 mutant V593.2 showed moderately reduced lesion development and a slightly slower growth rate was discernible as a smaller colony size after 3 days growth on minimal medium. V450 and V450.2 both exhibited delayed onset of symptoms compared to SAM116R in 100% of seedlings tested, but no noticeable variation on any of the plate assays. V421 and V502.2 also showed reproducible delayed onset of lesions, but no other observable differences in any of the characteristics screened. V41147 and V1150 also showed delayed symptom development combined with atypical lesions beginning with watersoaking in patches throughout the length of the leaf. As with V421, V450, V450.2 and V502.2, no other discernible variations from wild type behavior were observed. With the exception of SB41026, all mutant strains produced typical hypersensitive response on tobacco.

3.4 Discussion and Conclusion

Replication of ColE1, R6K and pACYC replicons was not supported by *X. campestris* pv. *zeae*. Broad host range groups P and Q replicons were maintained. RP4, although in itself mobilizable at high frequencies, failed to yield high levels of transfer of Tn5 containing vectors when the *mob* and *tra* functions were applied in *trans*. The various cloning vectors, however, including pGP704, when mobilized with SM10(λ*pir*) yielded high transfer efficiencies. In addition to RK2 replicons, the pSA based incW pUFR series replicons were maintained to high levels. P-*tra* based transfer systems were found to be highly efficient in *trans*, and cloning vectors able to use pRK2013 as helper plasmid were preferred due to high mating efficiency and relatively small vector size. Vectors tested and found superior were pLAFR1 and pDSK519. The apparent discrepancy that arises between P group transferred Tn5 delivery

vectos and the P-*tra* based cloning cosmids and plasmids must therefore be due to Tn5 functioning in the host and not due to the delivery system. This however does not explain why N-*tra* systems delivered Tn5 so efficiently.

Suicide cloning vectors based on the narrow host range E1 and p15A replicons and containing the RK2 *mob* were shown to be transferred efficiently and to insert by homologous recombination in cases where homologous sequences had been cloned into them. These vectors were construced with the aim of inserting cloned and altered suspected pathogenicity or virulence factors, and using these for functional gene disruption by homologous recombination.

The failure to transfer the P *mob* Tn5 delivery systems pSUP2021 and mini-Tn5-Tc, dictated the use of the N-*tra* system. The pGS9 Tn5 delivery vector, containing the N-*tra* system of pCU1 cloned into the pACYC184, p15A replicon, yielded the highest level of vector transfer and subsequent transposition. MJ Daniels (cited in Iyer, 1989) and Turner *et al* (1984) reported inefficient transposition of Tn5 using pGS9 as a delivery system into *X. campestris* pv. *campestris,* a finding which is in direct contradiction with results observe in pathovar *zeae*. Several other workers have used pGS9 with relative success, particularly in *Pseudomonas* and *Rhizobium* species (Iyer, 1989).

Higher donor:recipient ratio's were shown to increase efficiency of mating. At very high levels however, efficiencies did not increase proportionally, due to masking of recipient cells during mating. The reduction in relative efficiency at high cell densities corroborates this conclusion. Optimum mating conditions for N-*tra* systems functioning in *cis* were determined to be a ratio of donor:recipient of 3:1 and cell densities not exceeding 10^8/filter. To accomplish this cultures should be diluted accordingly and matings should not be allowed to proceed for longer than eight hours, which also prevents the isolation of multiples of the same organism.

Desired mutants were recovered at frequencies of 4.98×10^{-4} for nitrous acid mutagenesis and 1.53×10^{-3} for Tn5 mutagenesis. This is fairly typical in terms of mutant recovery. For example, isolation of *hrp*$^-$ mutants should occur at a frequency of 6.25×10^{-3} assuming a genome size of 4×10^3 Kb, random transposon insertion, and a *hrp* cluster plus *hrpX* size of 25Kb. SB41026 was recovered at 1.9×10^{-4}, a variation either due to limited data for accurate

statistical analysis, or indicative of a variation in *hrp* gene cluster characteristics. It must be noted, however, that the frequency of mutant isolation is only valid for screening for one genotype alteration. Similarly, A204 was recovered at 2.5×10^{-4} for protease activity representing a few kilobases in functional genes and export proteins, with additional possible regulatory elements - possibly as much as 15Kb, representing a fraction of the genome of about 3.75×10^{-3}. A section of the genome of approxiamtely 1Kb would have to be responsible for the regulation of protease production, the enzyme itself, and the export thereof to achieve a frequency of 2.5×10^{-4} for isolation of protease deficient mutants even if using a single site mutagenesis system which is substantially less likely to mutate a given sequence than chemical mutagenesis. The anomaly is enhanced when the consideration of all enzymes and colony characteristics are considered, and the total genetic material for the combination of phenotypes represents easily in excess of 1×10^{-2} of the genome, if only a portion of all the relevant metabolic determinants of chemoheterotrophy and chemoautotrophy are considered.

Since the frequency of recovery of mutants showing altered behaviour *in-planta* was in fact 1.5×10^{-3} for transposon mutagenesis, and assuming completely random Tn5 insertion, it may be deduced, based on mutant numbers, that a fairly small percentage of the genome plays a role in *in-planta* bahavior and virulence. Taking into consideration the percentage of the bacterial genome in which any mutations would be lethal this percentage may increase, but the low number is still indicative of possible non-random Tn5 insertion, particularly if the argument used to show the low recovery of chemical mutants is considered with the fact that exconjugant::Tn5 strains were screened for at least six distinguishable phenotypes representing a minimum of 1% of the genome. It is, however, possible that the relatively high inoculum used did preclude the isolation of many mutants with reduced virulence, as this has been shown to be a factor (Kao *et al*, 1992) despite the minimal effect of inoculum concentration on wild-type behavior. Denny *et al* (1990) showed that inoculation strategy, as well as other environmental factors were also shown to be significant, with natural inoculation strategies significantly enhancing the reduction in virulence in *Pseudomonas solanacearum* mutants deficient in one or two polysaccharidases. Similar results were obatined by Dow *et al* (1990) with varying methods of inoculation of protease mutants of *Xanthomonas campestris* pv *campestris*.

In conclusion, both chemical and transposon mutagenesis were found to be successful. Replicon recognition and replication, and inter-genus transfer were studied and appropriate replicons chosen for cloning and suicide purposes. A suitable suicide cloning vector was constructed and tested. Two suicide cloning vectors for insertional inactivation by homologous recombination were shown to work. Matings for transposon mutagenesis were optimized and factors effecting efficiency elucidated. A total of over 9000 mutants were generated and screened for several phenotype alterations. Ten mutants were isolated which showed alteration in one of the identified pathogenicity or virulence factors previously described. One apparently non-pathogenic strain, one *hrp*$^-$ mutant, a mutant deficient in protease exoenzyme activity, and seven mutants showing altered *in-planta* behavior, and reduced virulence.

Chapter 4 - Mutant Analysis

4.1 Introduction

5231 Km^R exconjugants and 4011 nitrous acid mutagenized isolates were purified and screened for any alteration in the production or export of any of the enzymes discussed, and for any alteration in pathogenicity or virulence, or in the ability to elicit HR on tobacco plants. In addition to these, over 3000 mutants were screened directly for alterations in protease and pectinase production. Direct screening for pectinase production was done by lifting colonies on sterile filter paper disks, prior to staining with congo red as previously described. One pathogenicity mutant (A204) was generated using nitrous acid, and one mutant (A3345) showing reduced protease production was also isolated after nitrous acid mutation. Tn5 yielded several virulence mutants, V421, V450, V450.2, V502.2, V593.2, V1147 and V1150 as well as one putative *hrp* mutant, SB41026. A204 proved to be a leaky mutant and not worth complementation analysis, while neither SB41026 or A3345 were recovered after a power failure resulted in the thawing of the cryo-storage tube in which the strains had been stored. The Tn5 disrupted genes from the virulence mutants were not cloned or analyzed since the mutations were considered to be essentially metabolic or nutritional in nature and not necessarily specifically pathogenicity or virulence gene related, as will be shown below.

4.2 Methods and Materials

Unless otherwise stated, the methods for culture and determination of culture characteristic, plant inoculation and enzyme assays, were as described in chapter 3.

4.2.1 Enzyme export, colony and growth characteristics of Mutants

The single partial protease activity mutant was subjected to further analysis to confirm the reduction in protease activity in liquid culture, using the plate well assay and TCA precipitation assays with casein as a substrate, as described for SAM116R in chapter three.

Growth characteristics were measured by monitoring optical density at 600nm in batch cultures with M9 minimal medium containing 0.5% glucose and Nutrient broth, regularly for 40 hours and 25 hours respectively.

4.2.3 Wild-type, SAM116R and Mutant *in-planta* behaviour

To minimize variation in *in-planta* behavior, only seedlings of comparible size, age and condition were used for pathogenicity and virulence testing. Inoculations were performed as previously described. Spread of bacteria was monitored by selecting three leaves every other day, and cutting two 20mm sections either side of the inoculation site, and one section containing the inoculation site. Where leaves were longer than the sites described, the remainder of the tip of the leaf was also sectioned as before, but with a maximum of 5 defined section, and the remainder of each end treated as a section. Each section was finely ground in a flamed pestle and mortar containing 5 ml sterile water. Serial dilutions were made and plated onto nutrient agar with no selection in the case of the wild-type, but containing 80mg/ml rifampicin for SAM116R and nitrous acid mutants, and the appropriate concentration of kanamycin in the case of Tn5 mutants. Lesions were measured daily on all plants remaining of a particular strain innoculum, with a minimum of five plants on the last day.

4.3 Results

4.3.1 Enzyme export, colony and growth characteristics of Mutants

As previously stated, none of the above mentioned mutant strains showed any quantitative variation in EPS production, pigmentation or colony morphology. None were auxotrophic, although some variation was observed in the growth rates of the organisms on both minimal and rich media. A single mutant A3345, showed altered protease production when assayed on milk agar plates.

4.3.1.1 A3345: a Mutant with Altered Protease Activity

The zones of clearing on culture plates of A3345 were substantially smaller than those of SAM116R. Diameters of zones of clearing for A3345 were between 4 and 7mm, with an average of 6mm. When expressed as a percentage of colony diameter, SAM116R had a zone diameter of 100%, while A3345 had a zone of approximately 60%. The higher level of variation in zone size in the mutant strain was attributable to colony size. Results of well based assays, where supernatent volumes were corrected to compensate for cell numbers, zones were consistently 40% smaller for A3345, as long as both strains were assayed in mid to late log phase. In early log, and stationary phase, A3345 and SAM116R respectively showed

relatively higher levels of activity. Figure 4.1 shows the variation in protease activity, as determined by well diffusion assays.

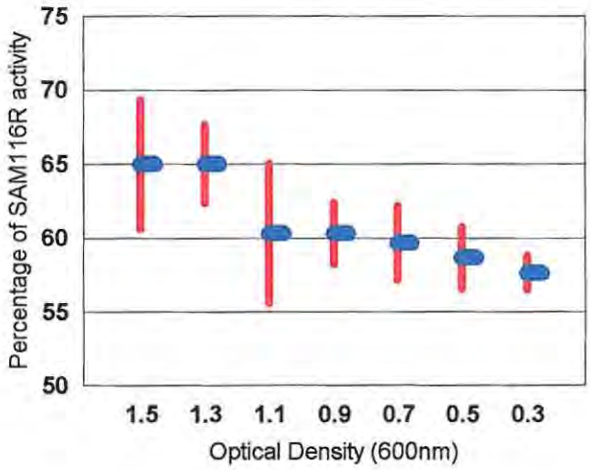

Figure 4.1. Percentage of protease activity observed in A3345 when compared to SAM116R at various phases of growth in batch culture. All extracts were concentrated and diluted to correct for cell densities. The trend in the average proteolytic zone diameter (horizontal lines) is apparent despite the relatively large standard deviation (vertical bar) obtained in the plate well assay, and appears to be as a function of cell density.

4.3.1.2 *In vitro* Growth of Mutants

Figures 4.2 and 4.3 show the *in vitro* growth of all isolated mutants, which on screening, repeatedly and reproducibly showed altered virulence, pathogenicity or nutritional features, on nutient broith with 0.5% glucose, and M9 minimal medium with 0.5% glucose respectively. With the exception of A204 (the leaky pathogenicity mutant) and A3345 (reduced protease), all mutants showed a significantly reduced growth rate, to a greater or lesser extent, in the rich medium. Mutant strains V1150, V1147, V421, V593.2, V502.2, and possibly V450.2 appeared to group together in terms of growth rates relative to SAM116R. V450 and SB41026 (the putative *hrp* mutant) possessed an intermediate growth rate. In glucose supplemented minimal medium however, four of the eight mutants showed growth rates similar to SAM116R, with the exception of V1147, which had a greatly enhanced rate of growth, V450, which grew significantly faster than SAM116R, while V593.2 and SB41026 had a much reduced growth rate. The addition of casein hydrolyzate to M9-glucose medium, at concentrations of 0.5% and 1% (w/v), although increasing the final cell concentration, had no significant effect on the growth rate of SB41026.

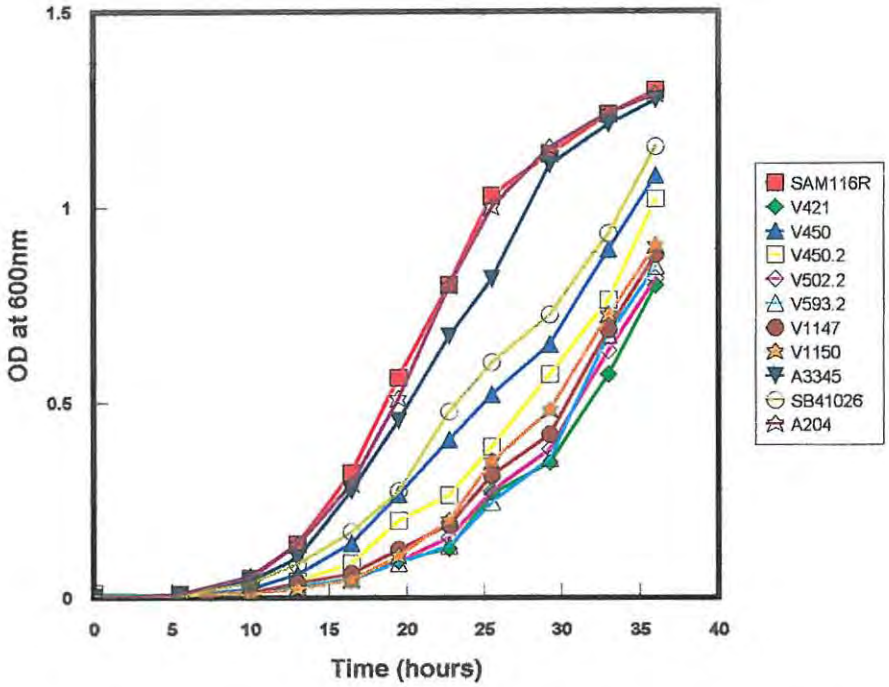

Figure 4.2 Growth in nutrient broth + 0.5% glucose at 28°C and 180rpm. All innoculums were standardized from fresh overnight cultures, and inoculated into pre-warmed nutrient broth at a ratio of 10^9cells into 200ml in a 500ml Ehrlenmyer flask. 1ml samples were taken every five hours and the absorbence read at 600nm. Results shown are averages of three cultures.

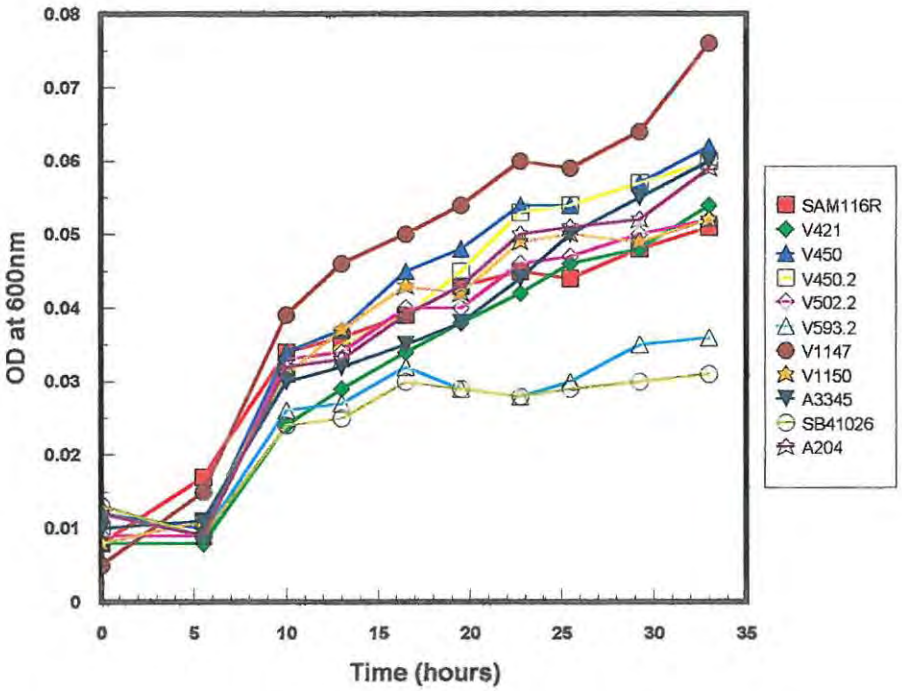

Figure 4.3 Growth in M9 minimal medium at 28°C and 180rpm. All inoculums were standardized from fresh overnight cultures, and inoculated into pre-warmed M9 at a ratio of 10^9cells into 100ml in a 500ml Ehrlenmyer flask. 1ml samples were taken every five hours and the absorbance read at 600nm. All points are averages of three cultures.

4.3.1.2 *In-planta* Behavior of Mutants

Figures 4.4 and 4.5 show the *in-planta* growth rates and lesion development for all mutants. Clearly *in-planta* growth fell into three major groups; i) wild-type-like, ii) slower than wild-type and showing reduction in cell numbers from 10 days, and iii) initial reduction in cell numbers with dramatic increase after 7 days (SB41026). The expected delay in increase in cell numbers as a result of a period of migration through parenchyma tissue towards vascular bundles was notably absent, indicating a rapid migration or substantial growth in non-vascular tissue. The rapid spread, only possible via vascular tissue, of SAM116R seen in figure 4.7, would indicate that migration to vascular bundles is rapid, and that growth does therefore not necessarily occur outside of xylem and xylem parenchyma cells.

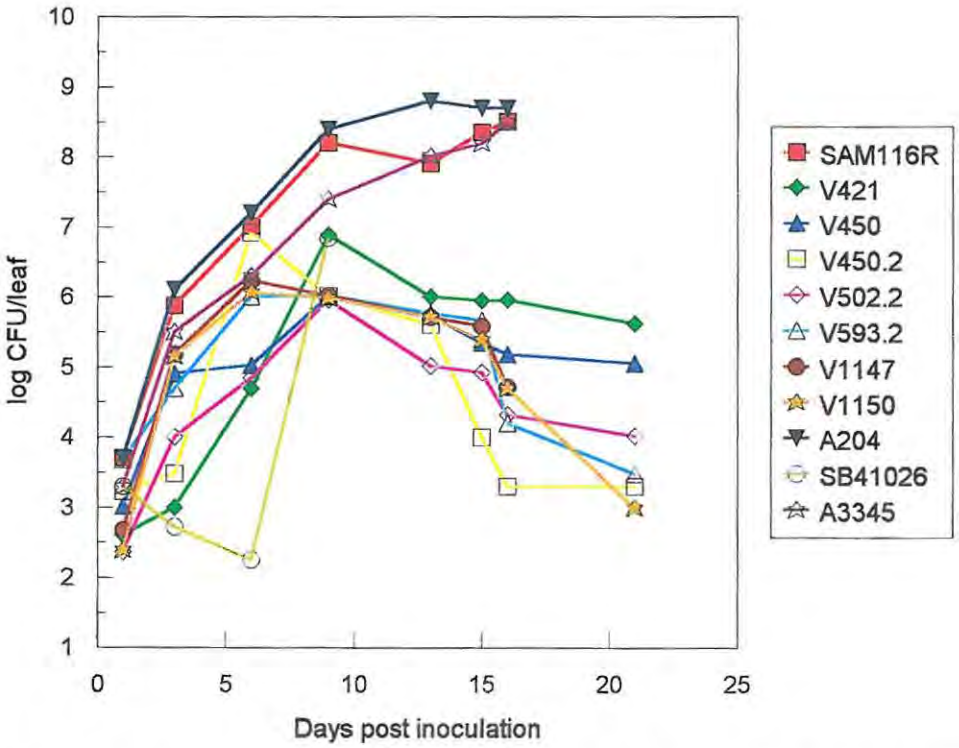

Figure 4.4 Growth in whole leaf after inoculation with the various Tn5 mutants and SAM116R. Figures are an average of 5 leaves. Data ends where leaves were completely wilted and were drying .

Lesion development fell into four basic categories; i) wild-type-like but slower (V421 and V593.2) , ii) late onset - small lesions (V450 and V450.2), iii) increased or normal lesion length with delayed symptom development (V1150, V1147 and V502.2) and iv) no lesions. Figure 4. 6 shows typical and atypical lesion development in maize seedlings.

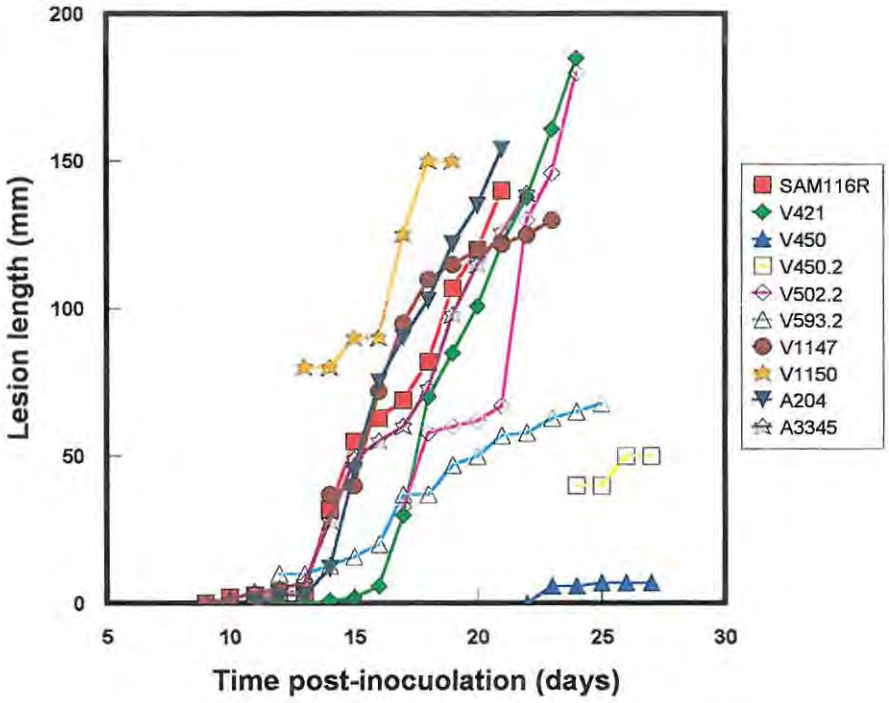

Figure 4.5 Lesion lengths as measured on inoculated plants daily after the onset of weatersoaking. All figures are the average of all available plants (minimum = 5).

All mutants produced typical HR on tobacco with the exception of SB41026 which failed to yield HR on tobacco at concentrations below 1 x 10^{10} CFU/ml, and did not produce lesions within the time period of monitoring. *In-planta* growth of SB41026 was as expected for a *hrp*⁻ organism, with a gradual cell number in the leaf post inoculation, but with a dramatic increase in cell numbers throughout the length of the leaf occurring after seven days (see figure 4.7), but still without the occurrence of lesions. The same pattern of reduction of cell numbers at the site of inoculation was observed with all mutants and SAM116R, although in the majority of cases this was short lived and was clearly as a result of cell migration away from the inoculation site, but was accompanied by substantial growth. SB41026, in contrast showed a reduction in cell numbers at all points, implying limited spread combined with a reduction in cell numbers at the inoculation site. Although these results are to be expected from a *hrp*⁻ strain, it must be noted that spread had to have occurred since growth in all leaf sectors resulted. The very low cell numbers obtained in leaf section adjacent to inoculation points (<100CFU/2cm) were therefore practically significant despite being statistically insignificant. The dramatic increase in cell numbers within the leaf was not expected, and is atypical for *hrp*⁻ organisms. It must however be noted that the innoculum used was particularly high, and the reduction in cell numbers, presumably due to host defences at the site of inoculation, may have

prevented any growth if a smaller innoculum more comparable with natural conditions was used.

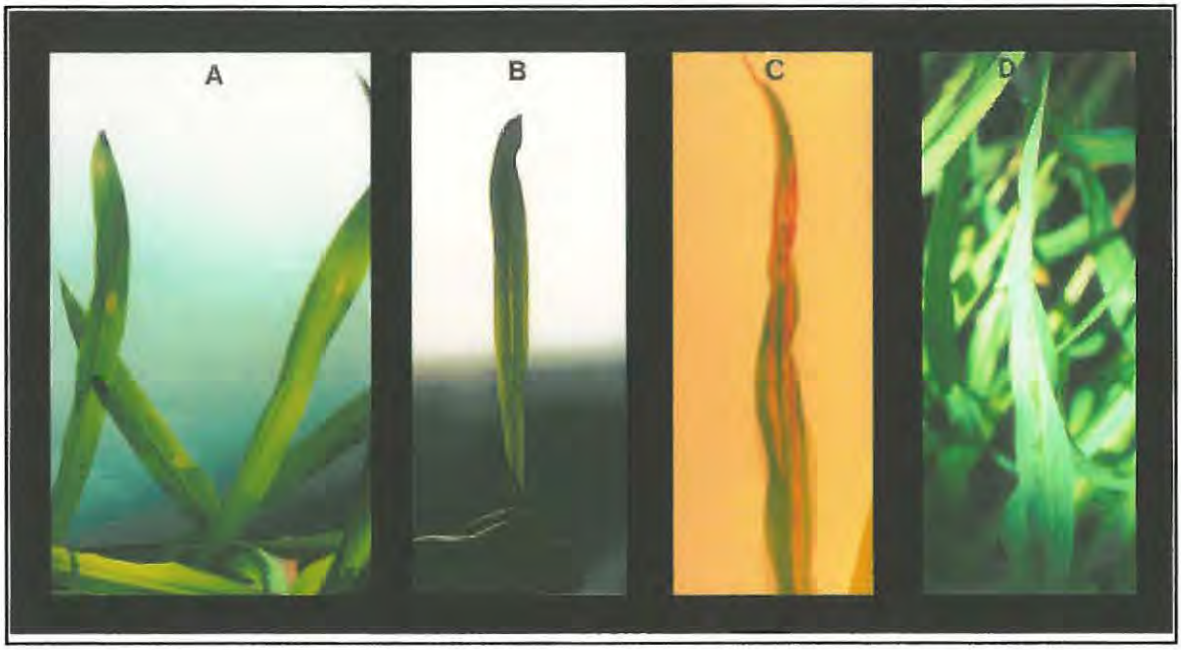

Figure 4.6 A series of photographs taken during one lesion development experiment. (A) to (C) show the progression of typical symptoms observed after inoculation with SAM116R (A) Typical initial symptoms caused by SAM116R after 1 week (B) Increased watersoaking, chlorosis and some necrosis in more advanced SAM116R infection after two weeks. (C) Typical extensive necrotic lesions in SAM116R infected maize after three weeks. (D) Atypical, extensive, noncontiguous watersoaking in a V1147 infected leaf after two weeks.

In-planta spread of the various strains, expressed as percentages in each leaf section of the total cells in the leaf (See figure 4.7), yielded four basic types of spread; i) wild-type like (including A204 and A3345), ii) reduced or delayed movement away from the site of inoculation (V1147 and V1150), iii) Movement predominantly in one direction but reduced or delayed (V421, V502.1, V450 and V450.2), and abnormal spread with abnormal ratio's (V593.2). Where ratio's of cells in leaf sections were as for the wild-type, the spread was considered normal. Where ratio's differed, spread was considered atypical. Where ratio's were similar but rates of achieving these varied, the rates of spread were scored as increased or decreased (See table 4.1)

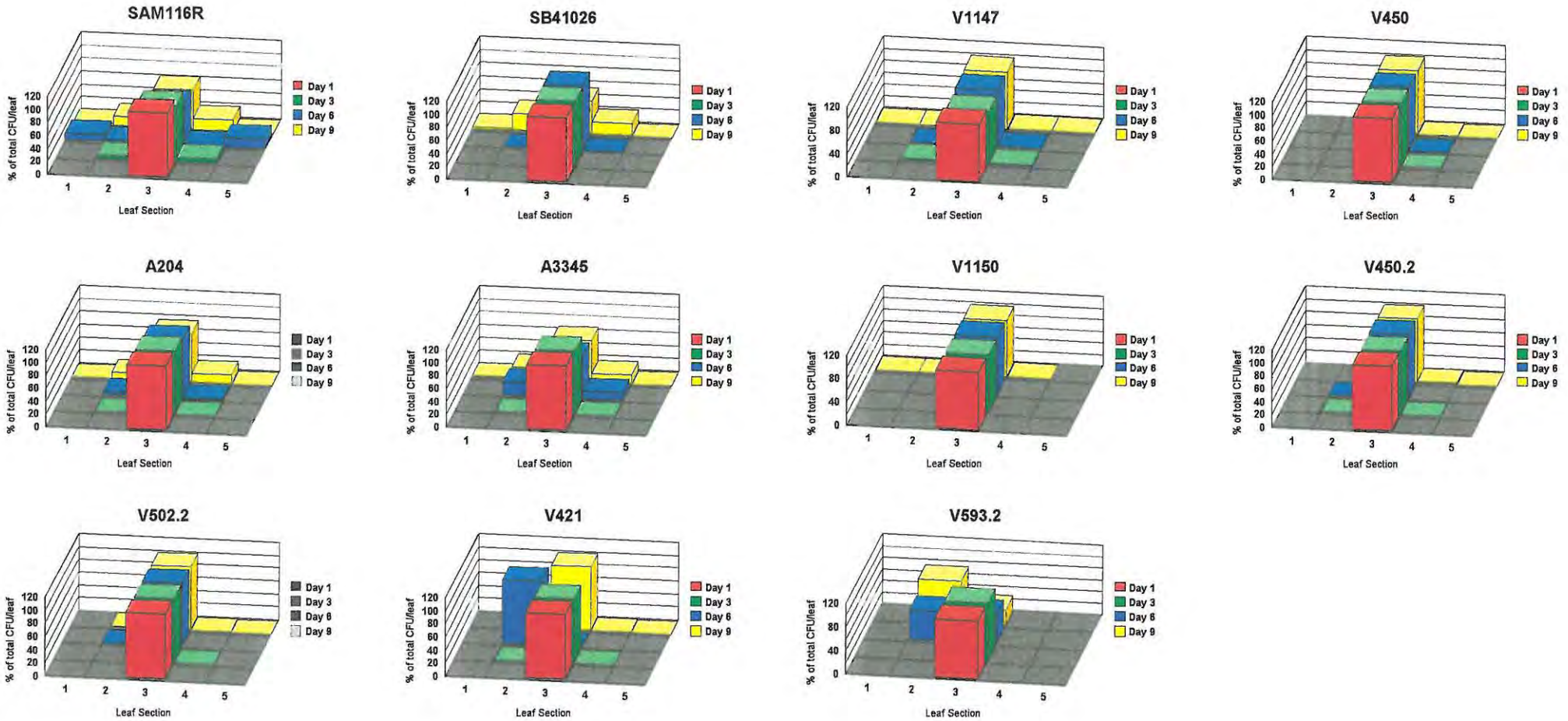

Figure 4.7 *In-planta* spread of all mutants and SAM116R expressed as a percentage in each 2cm leaf section, with the inoculation site in the centre of section 3, and section 5 being nearest the leaf tip

What may not be immediately evident from figure 4.7 is the immediate reduction in total cell numbers observed in SB41026 experiments, which is evident in figure 4.4. Figure 4.8 shows how this reduction is related to spread of the bacterium, with almost no spread occurring as the bacterial numbers are reduced.

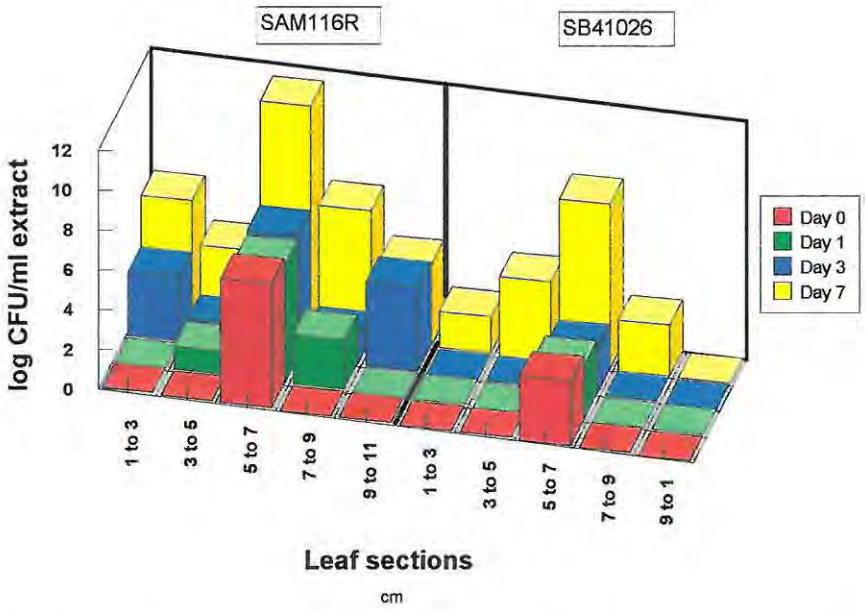

Figure 4.8 A comparison of the *in-planta* growth and spread of SB41026 and SAM116R.

The reduction in cell numbers after inoculation is typical of a *hrp* mutant. The subsequent growth is not typical but may be due to the relatively high innoculum which resulted in some spread despite cell reduction presumably due to host defenses, or possibly due to revertants. The reproducibility of this trend make the latter option unlikely.

In all cases where lesions developed as a result of infection by SAM116 or SAM116R, a threshold number of cells was required per leaf length before lesions developed (See figure 4.9) For SAM116R, V450.2, V421 and A204, the threshold was determined to be approximately 5×10^5 CFU/mm leaf tissue. Threshold figures for A3345 were higher on average, with approximately 6.5×10^5 CFU/mm leaf tissue being required for lesion development. Substantial variation however occurred in determination of these figures (Std. deviation 6.23×10^4 for SAM116R and 6.52×10^4 for A3345) tempering the conclusion that the reduction in protease activity resulted in reduced virulence.

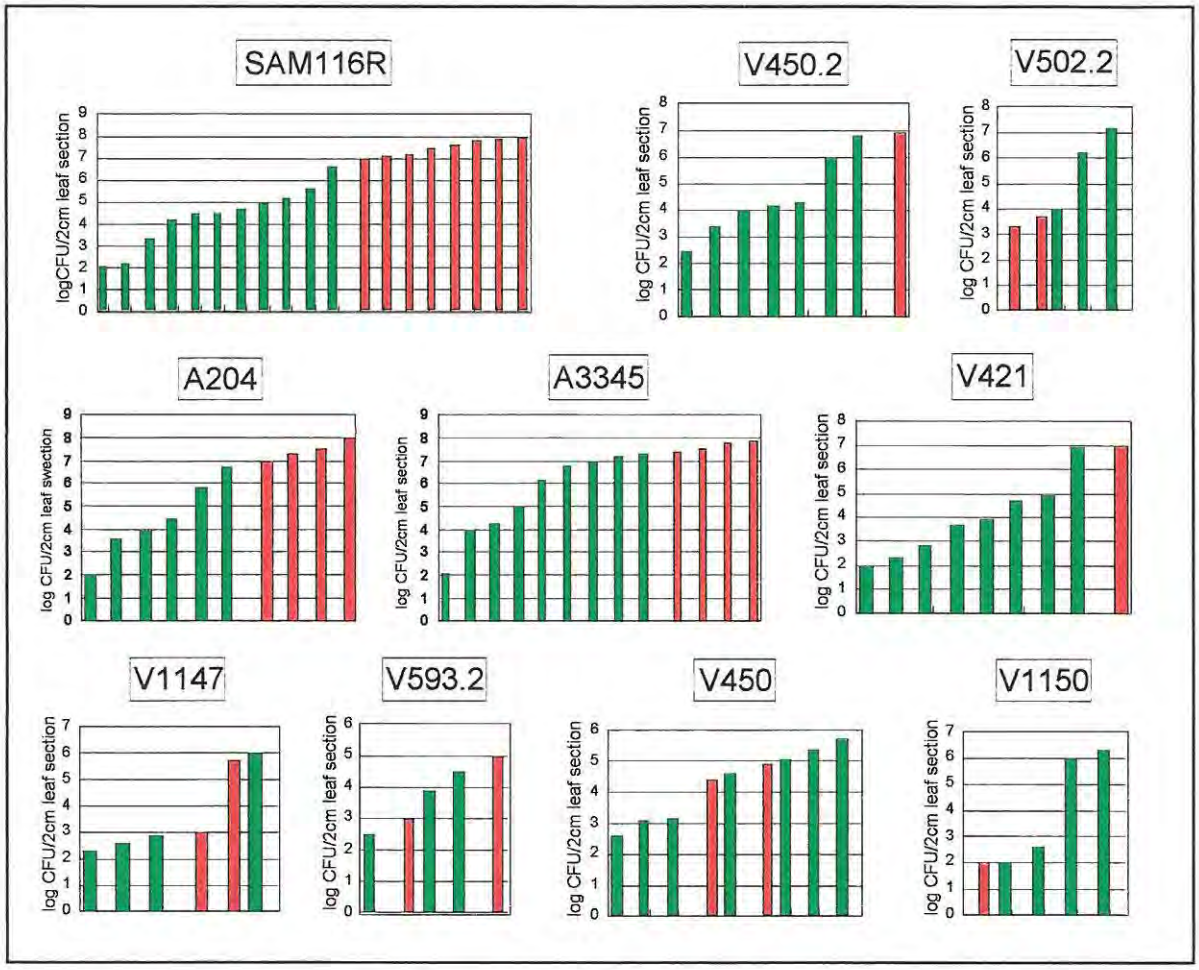

Figure 4.9 Threshold values for lesion development. Red bars indicate the presence of a lesion and green the absence thereof. Lesions were scored as positive if any sign of watersoaking, chlorosis or necrosis was visible to the naked eye.

V502.2, V1150, V1147, V450 and V593.2 lacked the threshold requirement for lesion development, with atypical lesion development at low cell numbers and absence of lesions at cell numbers approaching the wild-type threshold. Of particular interest, however, are the facts that no consistency in results was observed, and that in almost all cases these mutants failed to obtain cell numbers high enough to yield symptoms, and yet managed to do so. If the mutants had behaved as the wild-type in terms of threshold requirements, they would not have produced symptoms and would have been classified as non-pathogenic, particularly in the light of the reduction in cell numbers *in-planta*. For all these strains, lesions develop atypically in terms of cell numbers in a given segment of leaf, prompting the conclusion that the absence of lesions is the norm irrespective of cell numbers, and that symptom development by these mutants is atypical but so frequent as to be indistinguishable at the high innoculums used.

Table 4.1 Comparison of relevant features of mutant strains to SAM116R.

	A204	A3345	SB41026	V593.2	V450	V450.2	A421	A502.2	V1147	V1150
Growth in rich medium	N	-<X<N	-	-	-	--<X<-	--	--	--	--
Growth in minimal medium	N	N	--	--	+	N	N	N	++	N
Colony characteristics	N	N	N	N	N	N	N	N	N	N
Enzyme deficiencies	0	P-	0	0	0	0	0	0	0	0
Growth *in-planta* (first week)	-	-	---	-	-	-	--	-	-	-
Growth *in-planta* (later stages)	+	-	+++	--	--	--	--	--	--	--
Nature of Spread *in-planta*	N	N	A	A	A	A	A	A	N	N
Rate of Spread *in-planta*	N	N	D	A	DA	DA	DA	DA	DA	DA
Extent of spread *in-planta*	+	N	N	--	---	--	+	+	N	+
Rate of lesion development (early)	0/--	-	0	--	0	0	0	0	0	0
Rate of lesion development (late)	0/--	-	0	--	---	---	N	N	N	++
Extent of lesion development	0/++	N	0	--	---	---	+	+	N	++
Threshold	0/N	+	0	A	A	N	N	A	A	A

Key: N = normal; A = atypical; 0 = none

+ = slightly increased; ++ = greatly increased, ; +++ = greatly increased

- = slightly decreased; -- = decreased; --- = greatly decreased

P = protease.; < = less than where X is relative value; / indicates two possible results

D = Delayed

4.4 Discussion

Table 4.1 shows the relative colony characteristics and growth of each mutant in rich and minimal medium and *in-planta*, as well as the relative rate and extent of symptom development. Unfortunately mutants were recovered at too low a frequency to allow for sufficient mutants to be recovered to confirm apparent trends in nutrition - *in-planta* growth, nutrition - pathogenesis/virulence, and *in-planta* growth - pathogenesis/virulence relationships, but sufficient evidence exists to postulate relationships.

4.4.1 The threshold cell density required for lesion development

The requirement for threshold levels of cell numbers for lesion development suggests that lesion development is as a result of xylem vessel occlusion, which is supported by microscopic analysis of infected leaf tissue where xylem vessels appear to be fairly full of bacterial cells. However, taking into account the extent of vascularization of maize leaves, and the extremely low cell levels representing the threshold (500000/mm) it seems unlikely that complete occlusion is the cause of symptoms, since even a liberal estimation of volume taken up by 500000 cells, amounts to approximately 100000 μm^3 per leaf cross section of (500μm thick) which has a cross section area of approximately 2×10^7 μm^2 yielding a total volume in the region of 10^{10} μm^3, meaning that xylem vessels could account for only 1/100000 the leaf volume in cross section. Since the central vascular bundle alone accounts for nearly 50% the cross section volume it seems unlikely that even in a 1μm section that the xylem vessels should account for only 1%, which would be the total bacterial volume. This does not, however, diminish the potential role of bacterial occlusion of xylem pits, and does not exclude the possibility of vessel occlusion by copious EPS production. However, since variations in EPS production were not observed *in vitro*, and were thus assumed not to occur *in-planta*, the significance of the variation observed between mutants seems not to support the latter possibility. The evidence against general bacterial occlusion includes the atypical cell numbers in symptomatic hosts infected with certain mutant strains which exhibited altered *in-planta* growth characteristics which in turn resulted in faster dissemination than growth. If symptom development is atypical in mutants showing absence of threshold cell number requirements, then these lesions could be as a result of localized regions where thresholds are reached (since none of these strains are capable of growing to threshold levels under normal high rates of spread as seen in wild-type and even SB41026) due to vascular constrictions or other localized

conditions. Since fairly large segments of leaf were tested, it was however not possible to determine whether localized high density aggregations were occurring, which seems likely give the wild-type behavior.

Irrespective of mechanism of pathogenicity, it is clear that it is substantially different from *X. campestris* pv. *campestris*, as observed by the lack of threshold requirement and the almost linear relationship of cell numbers to mean symptom rating as observed by Daniels *et al.*. (1984).

An alternative to xylem vessel occlusion is xylem vessel damage. This too is supported by microscopy, with vessel breakdown being observed in infected tissue. The lesions are more easily explained by this phenomenon, since the initial watersoaking and chlorosis are more consistent with tissue damage than water stress. If this is the case, then the question of thresholds for exoenzyme production/export is raised. The difficulty in obtaining protease from early phases of liquid culture lends support to this idea, but insufficient evidence is available to draw any conclusions. Dow *et al.* (1990) report no proteolytic exoenzyme activity in minimal medium or casamino acid supplemented minimal medium, but substantial activity in rich media supernatents. Although no cell densities are given, the substantially slower growth in these media could account for this and substantiate the cell density hypothesis.

Strong evidence for this concept comes from the disseminated and noncontiguous (along single vascular bundles) lesions produced by the slower growing mutants where spread occurred. If vascular occlusion were the primary basis for lesions, these would spread from the point of infection away from the inoculation site towards leaf tips fairly rapidly due to water stress. Since entry into the xylem vessels is thought to be via the xylem parenchyma cells, it is also possible that damage to these cells alters xylem functioning, but the movement of bacteria out of the xylem into parenchyma has not been shown and is far less likely, resulting in a failure to explain the atypical lesions, and leaving this as the primary method of pathology of the organism in doubt.. However, since lesions only occur in the proximity of threshold numbers of cells in wild-type, it seems that typical wilt type scenario is inappropriate.

Whether damage is a result of localized occlusion or localized aggregation and subsequent plant cell destruction remains to be proven, but evidence given here indicates that occlusion plays a lesser role than previously thought, and that vascular bundle damage by exoenzymes may be more significant than previously supposed.

4.4.2 Correlation between nutrition, growth rate and virulence

Four distinct classes of mutants, based on nutritional behavior, can be distinguished from table 4.1. Class I, of which SB41026 and V593.2 are representatives, Class II, containing the mutants V450 and V450.2, Class III containing V421, V502.2 and V1150, and Class IV which contains V1147. Class I showed slightly reduced growth in rich medium and substantially reduced growth in minimal medium. This indicates a deficiency in a metabolic process which is attenuated by growth on rich medium which is typified by auxotrophy and although these mutants were not auxotrophic, there behavior was such that it could be established that they had a requirement for complex organic nutrients. Unfortunately, since SB41026 was a *hrp* mutant, further comparison is not possible, particularly since SB41026 was generated using Tn5, and as such has only one to three insertionally inactivated sites. As previously stated, at the high innoculum levels used, V593.2 was inevitably pathogenic, and at natural levels could easily have emerged non-pathogenic, but the HR response was as for wild-type. It is therefore possible that the function which was altered may have been one which was regulated by the *hrp* genes and thus yielded a 'partial *hrp* altered phenotype'.

Members of Class II are clearly more comparable, showing slightly decreased growth in rich medium (with V450.2 having slower growth than V450), but increased growth in minimal medium when compared to the wild-type. No variation was observed in any colony characteristics or enzyme activities, but a slightly reduced growth in initial stages of infection was observed, followed by a fairly dramatic reduction resulting in an actual lowering of total cell numbers in an infected leaf. As a result of this, symptoms developed very late, and were much reduced in both rate and extent of development.

Class III mutants showed a similar reduction in growth rates in rich medium combined with normal growth rates in minimal medium. As with the class II mutants, growth *in-planta* was significantly reduced in both early and late stages of infection, but unlike the previous class, lesion development, although delayed, proceeded at a normal or even increased rate once

established. Of particular interest was the increase in extent of the lesions. By comparison of Classes II and II, it would appear that there exists a correlation between the ability to grow well in minimal medium and a reduced rate and extent of lesion development, since no other measured characteristics showed any difference between the two classes. Since growth to higher numbers at a faster rate would result in thresholds being reached and hence lesions developing, it appears that spread is enhanced by initial slower or at least normal growth in minimal medium which can be equated with the lower nutrient status of xylem vessels.

The class IV mutant has a similar *in vitro* growth characteristics as class II, but an even lower growth rate in rich medium and slightly higher growth rates in minimal medium. These minor differences in extent of growth variation may account for the dramatic differences observed in the rate and extent of lesion development. In both classes lesion development is delayed, but in class IV the eventual onset of symptoms is followed by a normal lesion development in terms of rate and extent.

The ability to grow rapidly in minimal medium conditions combined with a substantially reduced growth in rich medium, results in extensive lesions since the organism could survive without obtaining the threshold necessary for exoenzyme synthesis and hence can spread in low numbers in the xylem vessels until such time as localized aggregations allow for the threshold to be reached and hence the noncontiguous disseminated lesions observed with these mutants.

4.4.2.1 What are the primary affectors of spread *in-planta*?

Reduced movement away from the sites of inoculation, coincides with reduced growth during the latter stages of infection suggesting that the mutants exhibiting this behavior could grow at normal rates *in-planta* but that growth was limited possibly due to the apparent inability to migrate away from the site of inoculation and subsequent nutrient depletion in these areas. This, however, fails to explain the eventual spread of mutants V41147, V41150, V421 and V502.2, all of which show normal, or increased, if somewhat delayed rate, and extent of lesion development. This is suggestive of growth in parenchyma tissue rather than xylem vessels, reducing spread. Microscopic analysis, however, abrogates this. SB41026, shows the same behavior when growth is considered, but with an immediate reduction in numbers, but also the increased extent and rate of spread in later stages (without lesion development however). It

would therefore appear that growth is not the primary factor affecting spread, but that increased growth and decreased growth, increase the rate and extent of *in-planta* spread respectively.

4.4.3 The Mutants

4.4.3.1 The growth mutants

All growth mutant characteristics were discussed in section 4.4.2 Tharaud *et al.* (1994) showed correlation between *in-planta* growth rate and virulence for *Erwinia amylovora*, which was similar in principle to that described here, with *path*⁻ mutants showing dramatically reduced growth, while those with reduced virulence showed initial reduction and then some growth at reduced rate. This is, however, purely coincidental and has no bearing on mechanisms of pathogenicity since these organisms are quite pathologically distinct. The evidence for correlation of growth behaviour and characteristics, both *in-planta* and *in-vitro*, and pathogenicity is, however, clear, and warrants further study. Certain difficulties will however plague such studies since the altered growth could be either cause or effect, the latter case being exemplified by the non-pathogenic mutants of *X. campestris* pv.*glycines* characterised by Hwang *et al.* (1992).

DE Crecy-Lagard *et al.* (1990) isolated a protein similar to *E. coli* K-12 catabolic activator protein (CAP) or cAMP receptor protein (CRP). A mutant with defective CLP (CAP-like protein) had strongly reduced pathogenicity but the utilization of various carbon sources was not affected. Other pathogenicity factors were however either increased or decreased suggesting a role for CLP in phytopathogenicity. Cloned *clp* was able to restore carbohydrate catabolism by a *cya crp* strain of *E. coli* suggesting a role not only in regulation of pathogenicity factors, but also in the growth behaviour of bacteria *in-planta*. The differential mutant growth observed in virulence mutants of *X. campestris* pv. *zeae* could be attributable to such differential carbohydrate utilization, but since many such catabolic systems are plant-inducible, this remains speculation. A similar problem in analysis of VsrA/VsrD growth mutants of *Pseudomonas solanacearum* by Huang *et al.* (1995), where mutations in a component of a complex environmental sensing and regulatory system were the expression of the primary pathogenicity factor (*eps)* was dependant on growth *in-planta*, a condition not unlike that observed in the virulence mutants described in section 4.4.2.

4.4.3.2 Protease deficient mutant

The increased threshold requirements for initiation of symptom development by A3345, although not dramatic, are significant. Since lesion development, along with *in-planta* growth rate are also retarded, although to a lesser extent and with lower significance, it seems likely that the decreased proteolytic activity is responsible for this reduction in virulence. The extent of lesions is not affected, but the rate of symptom development is. Since rate of lesion development is directly related to cell numbers in a specific leaf segment, assuming normal threshold requirements for the strain, and hence *in-planta* growth rate, this is not unexpected and may be attributable to the reduction in protease activity which would correlate with results found for *X. campestris* pv. *campestris* (Dow *et al.* 1990; Tang *et al.* 1987), tempting the conclusion that this is so. However, the tissue maceration symptoms produced by *X. campestris* pv. *campestris* are distinctly different to the chlorotic and subsequent necrotic lesions accompanied by wilting that are observed with the maize specific pathovar. That the reduction in virulence was minor in this mutant confirms this. In addition to these problems, the extensive mutagenesis produced by nitrous acid treatment could easily have resulted in nucleic acid lesions other that the one/s responsible for the reduction in protease activity, and possibly responsible for the reduction in virulence. Interestingly, in addition to the protease deficient mutants of *X. campestris* pv. *campestris* produced by Tang *et al.* (1987) showing a similar slight reduction in *in-planta* growth when compared to the wild-type, the observed growth pattern of the wild-type was similar, with an initial increase, a dip in cell numbers and subsequent increase in growth again. Initially this was thought to be as a result of entry into vascular tissue, but the same result in pathovar *campestris* precludes this conclusion and points to an alternative cause.

4.4.3.3 The putative *hrp*⁻ mutant SB41026

SB41026 was clearly unable to induce HR and probably *hrp*⁻ despite the delayed higher levels of growth observed in maize seedlings. The absence of any HR on tobacco, combined with the reduction in cell numbers and lack of symptoms on a compatible host verify this. The increase in cell numbers accompanied by spread *in-planta* are anomalous and was probably due to the relatively high innoculum used to ensure reproducibility (see section 4.2).

4.4.3.4 The pathogenicity mutant A204

A204 showed wild type level exoenzyme production, typical colony characteristics and HR on tobacco, and normal growth in both rich and minimal medium. The only observable characteristics showing any variation occurred *in*-planta, with an initial reduction in growth rate and a later increase in growth relative to SAM116R. In 15.5% (13 out of 84) of cases, seedlings inoculated with A204 did yield symptoms. Where symptoms did develop, the lesions were delayed in onset but more extensive and faster developing, as would be expected with an initially slower growth rate allowing faster spread, followed by faster growth allowing thresholds to be reached. Since A204 only showed these variations *in-planta* the mutation was clearly pathogenicity related. Daniels *et al.* (1984) describe this complete but occasional pathogenicity as "leaky", and speculate that this is a result of mutation in one or a few of the necessary pathogenicity factors resulting in a reduced probability of infection. This does not, however, explain the fact that infection occurs readily but even at wild-type threshold levels, no symptoms develop. Clearly additional pathogenicity factors over and above plant cell wall degrading enzymes and amounts of EPS, and in addition to any modifications in growth rates under both rich and minimal conditions, and not under control of the *hrp* genes exist. The leaky nature of the mutation may be attributable to a combination of cell density and the A204 specific DNA mutation, or alternatively to reversions which are induced *in-planta*.

4.5 Conclusion

Insufficient mutants were isolate to fully understand the relationship between growth requirements and virulence. It was however apparent that, to maintain virulence in the host the bacterium must balance growth *in-planta* with spread which, in xylem vessels, is a passive function. This being the case, the primary factor which effects virulence is the relative growth rates under rich and minimal conditions. Enhanced growth under conditions similar to those in the host plant, results in substantially reduced virulence as measured by rate and extent of lesion development if the bacterium's ability to grow in rich medium has not been reduced dramatically. If significant reduction in growth under rich conditions has occurred, and growth under minimal conditions is much enhanced, normal lesions occur but are delayed in onset. This is presumably as a result of the organisms balanced growth *in-planta* as function of the ability to utilize complex organic nutrients to a lesser extent, but able to grow faster under minimal conditions and hence to approach wild-type growth dynamics *in-planta*. This appears to be confirmed by the observation that reduced utilization of complex organics, coupled with

normal growth in minimal medium, are characteristics of strains that yield extended lesions at a normal rate after a delay in onset, as a result of growth being balanced by spread, but in low numbers initially.

Chapter 5 - Final Discussion and Conclusion and Future Work

5.1 Final Discussion and Conclusion

Xanthomonas campestris pv. *zeae* was shown to posses a vast range of potential plant cell wall degrading enzymes including at least one protease, a carboxymethylcellulase, pectin lyase, polygalacturonase and a *B*-endoglucanase. Slight avicellase and potential cellobiohydrolase activities were also observed. Replicon stability and transfer system efficiencies were determined for a range of *ori*C, and *ori*T -*tra* combinations, and suitable vectors were constructed and identified for insertional inactivation by homologous recombination. Ideal suicide replicons were found to be pACYC184 and p15A, while P, W and Q group replicons were supported by *X. campestris* pv. *zeae*. Group P and group N transfer systems were shown to be highly efficient in intra-genus matings between *Escherichia coli* and *X. campestris* pv. *zeae*, with the exception of P-*tra* systems in *trans* for the delivery of Tn5. Various cloning vectors were tested for stability and mobility, and the behaviour of Tn5 in *X.* campestris pv. zeae was established. Tn5 was shown to transpose at high frequencies into the genome of the bacterial plant pathogen, and insertion was random. Suitable screening assays were established to allow rapid isolation of mutants with potential virulence or pathogenic deviations, after mutageneis.

Ten mutants were isolated. Nitrous acid mutagenesis yielded extremely low levels of mutants for the phenotypes screened, probably as a result of extensive mutagenesis working to reduce rather than enhance generation of desired mutants. Transposon mutagenesis yielded mutants at the expected frequency. A low frequency of enzyme deficient mutants suggested a lethal nature for these mutations, a postulate which in itself is highly unlikely and leads to speculation on possible upstream regulation. However, no such speculation could account for the inability to achieve simple enzyme insertions and hence single enzyme deficiencies. A low frequency of generation of non-pathogenic mutants was observed and was probably due to high stringency screening as a result of high innoculum levels and invasive inoculation techniques, since the maize line reported to

be resistant was highly susceptible when this type of inoculation was used. Apparently breeding for resistance has selected for reduced surface openings, such as smaller stomata, to prevent infection rather than eliminate bacteria in the host plant. Two non-pathogenic mutants were identified, one of which was judged to be *hrp*$^-$, while the other was unfortunately a leaky virulence mutant and hence not suitable for complementation analysis. A single mutant showing 40% reduced protease activity was also shown to be reproducibly less virulent. The limited extent of the reduction in virulence indicates a lesser role for the protease, or a lesser requirement for expressed levels of protease. Alternatively the protease deficiency was only significant *in vitro*, a possibility which was not nullified by the results obtained in protease induction experiments. The absence of Tn5 induced protease mutants could be explained if protease is *hrp* controlled, as is suggested by various *hrp* studies, then no mutants could be isolated, other than enzyme mutants themselves, unless a *hrp* mutant was isolated which had the specific regulatory mechanism inactivated, which would automatically lead to non-virulence and possibly pleiotropic export deficiencies possibly rendering such a mutant non-viable. Alternatively, export mutants could also be non-viable by the same token.

The majority of mutants showed altered growth in different levels of nutritional availability and complexity. Nutritional viability (the ability to acquire and use nutrients at a sufficient rate to grow fast enough to overcome host defences) was shown to be essential for virulence and possibly pathogenicity. Wild-type *in-planta* behaviour was analysed and growth and spread patterns typical for pathogenic response identified. Chief amongst these was the requirement for a threshold level of cells per leaf area or length, before symptoms could develop. Occlusion of vascular bundles was, however, shown in all likelihood not to be the primary factor in the pathogenicity of *X. campestris* pv. *zeae*, since mutants were isolated which were able to exceed the threshold for normal symptom development in the wild-type organism, while having normal EPS production. What the threshold levels rather indicate is the absence of a diffusable element responsible for lesions, and possibly the requirement of cell density for induction of certain functions.

5.2 Future Work

The use of *Xanthomonas campestris hrp* genes as probes for the *hrp* cluster of *X. campestris* pv. *zeae* will be investigated. In addition to this, primer sets for the *hrp* cluster as well as additional primers for the *hrpX* gene will be constructed and used to characterise these genes. Once cloned, selective insertional inactivation by homologous recombination with appropriate gene fragments, using the purpose built vector described, should begin to allow understanding of the functions of these genes in pathogenicity. In addition to the continuing analysis of known pathogenicity factors, results obtained during this work suggest a greater role for the growth rate *in planta* in the virulence of the organism, and investigation of the conditions resulting in this growth, in conjunction with *in-planta* enzyme expression work (envisaged once sufficient enzymes have been isolated to use for antibody production) should yield greater insight into the pathogenicity and virulence of *X. campestris* pv. *zeae*.

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Appendix I

Buffers and Solutions not described in text

Acetate buffer 0.1M pH 4.6
Solution A: 11.55ml glacial acetic acid in 1ℓ H_2:O
Solution B: 27.29g Na-acetate.$3H_2O$ in 1ℓ H_2:O
25ml Solution A added to 24.5ml Solution B and 40ml distileld water, pH adjusted to 4.6, volume adjusted to 100ml and autoclaved

Phosphate buffer 0.1M pH7
6.8g KH_2PO_4 + 1.16g NaOH in 480ml distilled water. pH adjusted to 7 with 2N NaOH, and volume adjusted to 500ml

5 x TBE buffer
54g TRIS base
27.5g Boric acid
20ml 0.05M EDTA

Gel Loading buffer
0.25% bromophenol blue
0.25% Xylene cyanol FF
30% Glycerol in distilled water

Appendix II

HrpXo and HrpXc alignments and *HrpX* primer positions in colour

```
HrpXc       gatatcaatactcaaccggatggcgcgaaatgtctggcgtacgacaatgccaacgatgtg   60
HrpXo       ------------------------------------------------------------    0

HrpXc       caaatgcgctttgcgtcgggagaaaacgcatcacatggttgtttgacagagtgagctttt  120
HrpXo       ------------------------------------------------------------    0

HrpXc       ttgatttttcaatggctttcagagggaaattctattGGATCCGCTGCATACAATCGTTT   180
HrpXo       -----------------------------------GGATCCGCTGCATACAATCGTGT    23

HrpXc       GCGCCAGCGAGTTCGGCGCCTGTTGTCTTTTGCTCCGCCCCCCAAaGAGAGAGACCGGCA  240
HrpXo       GCGCCACGGAGCTCGGCGATTGTTGTCTTTTGCTCCGCCCCCCAA-GAGAGAGACCGGCA  82

HrpXc       TGATCCTTTCCACCTACTTTGCAGCGATCTCTGCGTTGTCATACGCAGAACGTCTTCCTA  300
HrpXo       TGATCCTTTCGACCTACTTTGCAGCGATCTCTGCGTTGTCTTACGCAGAACGTCTTCCTA  142

HrpXc       CCTATACGAGCAGGATGCTGGTTGGTGCTTGGCCACAGGGACTGCAACATCTCCAACAGC  360
HrpXo       CCTATACGAGCAGGATGCTGGTTGGTGCTTGGCCGCAGGGACTGCAACACCTTCAACAGC  202

HrpXc       GACGCGACGGACAGGGCGCAGCGGATGGCGCCGACGATGAGGTCAGCTTGTTCGGTGCCA  420
HrpXo       GACGCGACGGGCAGGGTGCAGCCGATGGCGCCGACGATGAGGTCAGCCTGTTTGGTGCCA  262

HrpXc       GCGGCGATGCGTTGCTGATCCTGGAATATCAGGAAGAGGCCGAAGATGCGTATCGGCAGG  480
HrpXo       GCGGCGATGCGCTGCTGATCCTGGAATATCAAGAAGAGGCCGAAGACGCGTATCGGCAGG  322

HrpXc       CCTTGAAGGCCATGCGCGGGCATCAGCGCCAATTGCGCCTGCTGTCGTGCCGGAACACCG  540
HrpXo       CTTTGAAAGCCACTGCGCGGGCAGCAGCGCCAATTGCGTTTGCTGTCGTGCCGGAATACC  382

HrpXc       CCTGGTTGATGCTGAGACAGCGGCGCCTGAGCGCGGCGTTGAATTGCTTCGCGCAGTTGG  600
HrpXo       GCCTGGTTGATGCTGAGCCAGCGACGCCTGAGTGCGGCATTGAACTGCTTTCGCAGCTGC  442

HrpXc       CAATGGACCGCGAAACGCCGCCCAGCCTGTGCGGCGAGAGCCTGTTGGGCAAATCGCTCA  660
HrpXo       GATGGATCGCGAAACGCCCCCCAGCCTGTGCGGCGAGAGCCTGCTGGGCTCAAGGCACTG  502

HrpXc       CCCACTTCCATCTGGGCCAGAGCACGTTGGCCTTACAGACCCTGGAGCGTGCCCGCGAGG  720
HrpXo       ACCCACTTTCATCTGGGCCAGAGCACGCTGGCCTTGCAGACCCTGGAGCGCGCCAACGAG  562

HrpXc       TGCTGGCCGACCTGGAGAGCGGCGCGTCGGACTGGCTGCGGATTGCCACTGCGCTGCGTC  780
HrpXo       GTGCTGGCCGAGCTGGAGAGTGGTGCTTCCGATTGGCTGCGTCTCGCCACCGCGCTGCGG  622

HrpXc       TGGACATGATTGCGCAGCTGCGTATCCGTCGCTCCGACCGCATGAGCGACCATGTGTTCT  840
HrpXo       CTGGACATGATCGCGCACTGCGCATCCGCCGGTCCGATCGCATGGTCGACCACGTGTTCT  682

HrpXc       GGCAAGCCACGCTGCGCCAGGAAGATGCCACGCATGCCTTCGAGCCCAGCGACCTGCGCG  900
HrpXo       GGCAGGCCACGCTGCGCCAGGAAGATGCCGCGCATGCCTTCGAGCCCAGCGATTTACGCG  742
```

```
HrpXc       CCTGCATCGCCGATCTGGCAGAGAGCATGCCGGTGCTTGCGCAGCGCATGCGGCATGTAC  960
HrpXo       CATGCATCGCCGATCTGGCCGAGAGCATGCCGGTGCTGGCGCAGCGCATGCGGCATGTGC  802

HrpXc       GCAGCCTGTTGCGTATTGCCGGCGGCGACACCTTCGGCTTCGACGCGCAGATCGACGCAT 1020
HrpXo       GCAACCTGCTGCGGATCGTCGGCGGCGATACCTTCGGCTTCGATGCGCAGATCGATGCGT  862

HrpXc       TGCCGGCCGCAGCCACCGGCTGCCCGCCGTGTGCGCGTCAGGACGCGCAGGTGGAACTGG 1080
HrpXo       TGCCGGCTGCGGCCACCGGCTGCCCGCCGTGTGCGCGTCAGGACGCACAGGTGGAACTGG  922

HrpXc       CGCTGGCGGCATTGGCGGTGGGCCGTGCCGATCTGGCCGAGCGTGCGCTGGCCGGTGCCG 1140
HrpXo       CGCTTGCCGCATTGGCGGTGGGGCGTGCCGATCTGGCCGAGCGCGCGCTGGCCGGTGCCG  982

HrpXc       GCCGACATCATGCGCCGCGCTGGAACCTGGAGTTCGAGTACTGCCAGGCCAAGATCAGCC 1200
HrpXo       GCCGGCATCATGCGCCGCGCTGGAATCTGGAGTTCGAATACTGCCAGGCCAAGATCAGTC 1042

HrpXc       AGGCGATGGGGCGCACCGAACAAGCGTTGCTGCTCTACAACCGCTACGCGCTGGGCGCGG 1260
HrpXo       AGGCGCTGGGACGCACGGAACAAGCGTTGTTGCTCTACAACCGCTATGCGCTGGACGCGG 1102

HrpXc       TGCAGTGCCTGCGCAGCGAAGTGCAGGCACCGCGCTTGCTGGAAAGCGGCACCCCGGCGC 1320
HrpXo       TGCAATGCCTGCGTAGCGAAGTGCAGGCACCGCGCTTGCTGGAAAGCGGTACCCCAGCGC 1162

HrpXc       ACGTCAGCGACGATATCTCCGCCCGGTTGCCGGCCAAGTATCGGCGCGCCTACAGCTACA 1380
HrpXo       ACGTCTCCGACGATATTTCCGCCCGCTTGCCGGCCAAATATCGGCGTGCGTATAGCTACA 1222

HrpXc       TGATCACCAATGCGCATCGCTCGGATCTGTCGATCAACGAAGTGGCCGCACAGATCGGGG 1440
HrpXo       TGATCACCAACGCGCACCGCTCGGATCTGTCGATCAATGAAGTGGCCGCACAGATTGGGG 1282

            AGTGGCCCGCGCGCGACGA
HrpXc       TCACCGGGCGCGCGCTgcaGCAAGCGTTCAAGTCGGCTACCGGGCTATCGCCGACGCAGG 1500
HrpXo       TGACCGGGCGCGCACT---GCAGGCGTTCAAGTCGGCCACCGGGCTGTCGCCGACCCAGG 1339

HrpXc       TGCTGCGCCGCTACCGGATGCAAAGCATCCGCGACGAACTGCTGGCCGAAGGcgGCTGCG 1560
HrpXo       TGCTGCGCCGTTACCGCATGCAGAGCATCCGCGACGAACTGCTGGCCGAAGG--GCTGCG 1397

HrpXc       GCAGTGTGTTGCAGGCGGCCAGTCGCTGGGGCGTCGGCAGCCGTTCGGCGTTGGCCAAGG 1620
HrpXo       GCAGCGTGTTGCAAGCGGCCAGCCGCTGGGGTGTGGGCAGCCGTTCGGCATTGGCCAAGG 1457

HrpXc       GCTATCGCCAGCACTTCAATGAGGCGCCGATGGAGACCCTGCAGCGGTAATCTCcgcaac 1680
HrpXo       GCTATCGCCAGCACTTCAACGAAGCACCGATGGAAACCTTGCAACGGTAATCTCttggca 1517

HrpXc       ccacggattggtagcgctaccgatccgtggtgggtcaacccggcgtgcggtgcagtgcgc 1740
HrpXo       cgccacggcttggtagcgctacctgccgtgg---------------------------- 1548

HrpXc       gtcgctgtacgcaacggagggcaggggtcaaactgagacgtgcgtgcaatgtgggttccg 1800
HrpXo       ------------------------------------------------------------ 1548

HrpXc       gctagc                                                       1806
HrpXo       ------                                                       1548
```

Appendix III

Xanthomonas hrp genes with high similarity to *Pseudomonas solanacearum hrp* genes present in the clone pBS2.17

Comparison with *Xanthomonas campestris* pv. *campestris hrpB3*

Upper case shows region of highest similarity

```
pBS217      gcgcttgcgccgtggcgcgctggttgtcgcgctcgcgctgaccacGCTGCTGGCCGGCTG  6420
Xchb3       ----atgcgcgcgccgagatgcctggtggtgctgctggtggcgctGCTGCTGTCTGCGTG    56

pBS217      CAACAAGCAGCTCTTCGCCCAGCTGACCGAAGCCGATGCCAACGACATGCTGACCGTGCT  6480
Xchb3       CAGTCAGCAGCTGTACTCCGGGCTCACCGAAAATGATGCCAACGACATGCTCGAGGTGCT   116

pBS217      GCTGCAGGCCGGCATCGACGCGCAGAAGACCTCGCCCGACGACGGCAAGACCTGGTCCGT  6540
Xchb3       GCTGCATGCGGGCGTGGATGCGTCCAAGGTCACGCCAGACGACGGCAAGACCTGGGCGGT   176

pBS217      GCTGGTCGACGACGACGCCTTCGCGCGCTCGATGGAGGTGCTGCACGCCCACGGCCTGCC  6600
Xchb3       GAACGCACCGCACGATCAGGTGTCCTATTCGCTGGAGGTGCTGCGCGCGCATGGTTTGCC   236

pBS217      GCGCGAGAAATACGCCAACCTCGGCGACATCTTCAAGAAGGACGGCCTGATCTCCACGCC  6660
Xchb3       GCATGAGCGGCACGCCAATCTCGGCGAGATGTTCAAGAAGGACGGGCTGATCTCGACACC   296

pBS217      GACCGAGGAACGGGTGCGCTTCATCTACGGGGTGTCGCAGCAGCTGTCGCAGACGCTGTC  6720
Xchb3       CACCGAAGAGCGCGTACGTTTCATCTATGGTGTGTCGCAGCAGCTATCGCAGACCTTGTC   356

pBS217      GCGCATCGATGGCGTGGCGGTCGCCAGCGTGCAGATCGTGCTGCCCAACAACGATCCGCT  6780
Xchb3       GAATATCGATGGGGTGATCTCGGCCGATGTGGAAATCGTGCTGCCCAACAACGACCCGCT   416

pBS217      GGCCAGCGTGGTCAAGCCGTCCAGCGCTTCGGTGTTCATCAAGTACCGGCCGACGGCCAA  6840
Xchb3       GTCCACCTCGGTAAAGCCGTCCAGCGCTGCGGTGTTCATCAAGTTCCGCGTCGGTAGCGA   476

pBS217      CGTGACCGCGCTGCTGCCGAGCATCAAGAACCTGGTGGTGCACAGCGTGGAAGGGCTGAC  6900
Xchb3       TCTGACCAGCCTGGTGCCCAACATCAAGACGTTGGTCATGCACAGCGTGGAGGGACTCAC   536

pBS217      GTATGAGAACGTCGCCGTGACGCTGGTGCCCGGGACGGCGGAcgaccccgccatcgtggc  6960
Xchb3       CTACGAAAACGTCAGTGTCACGCTGGTGCCGGGCGGAGCGGAgagcgatgcgcagttcac   596

pBS217      cgccaccgcgcggcgcgccgcgcggcgtgtcgtgggtgatgctgggcagcctggccgg  7020
Xchb3       cgcatcggcaccgccgagaccttcgccatggccgtggctggccgggtgtgcgttggcgtt   656

pBS217      cgtgctggtcggcctggtggcgctgtggagcgcggtgacgcgcatgccggccgtgcgcac  7080
Xchb3       gtgcctggcgggcgcggcggcgctgtattggtggccaaacccgcaggcgggccgttgggg   716

pBS217      gcgcctggatgcgctgcgcgagcggatcgccagccgcatgccggcccgcctgaagaagcg  7140
Xchb3       cggctggcagcgcctgcgcgagttgaagaaaggcaaagccgggtga--------------   762
```

Comparison with *Xanthomonas campestris* pv. *campestris hrpC2 (Reverse orientation)*

```
Score = 2571 (710.4 bits), Expect = 5.7e-286, Sum P(3) = 5.7e-286
 Identities = 627/768 (81%), Positives = 627/768 (81%), Strand = Minus /
Plus

pBS217  7483 GATTTCAGCGGCGAAATCGGTATCGCCGCGCTAGTGGTGGCGGTGGTGGCGCTGATGGTG
XCHC2     37 GCTTATAGCGGCGAAGTAGCCATCGCCGCCCTGGTAGTGGCGGTCATCGGGCTGATGATC

pBS217  7423 CTGCCGCTGCCGACCATGCTGATCGACGCGCTGCTCGGTCTGAACATCACGCTCTCGGTC
Xchc2     97 CTGCCGCTGCCAACGCCCATGATCGATACGTTGCTGGGCATCAACATCACCCTGAGCGTG

pBS217  7363 GTGCTGCTGATGGTGACGATGTACATTCCGTCGGCGACCTCGCTGTCGGCGTTTCCGTCG
Xchc2    157 GTGCTGTTGATGGTCACGATGTATGTGCCCGACTCGATCTCGCTCTCGTCGTTTCCTTCC

pBS217  7303 CTGCTGCTGTTCACGACGCTGCTGCGGCTGTCGCTGAACATCGCCTCCACCAAGTCCATT
Xchc2    217 CTGCTGCTGTTCACCACGCTACTGCGGTTGTCATTGAATATCGCCTCGACGAAGTCGATC

pBS217  7243 CTGCTGCACGCGGACGCCGGCCACATCATCGAGAGCTTCGGCAAGCTGGTGGTGGGCGGC
Xchc2    277 CTGCTGCATGCCGAGGCGGGCCATATCATCGAGAGCTTCGGCGAGCTGGTGGTCGGTGGC

pBS217  7183 AACCTGGTGGTGGGCCTGGTGGTGTTCCTGATCATCACCACGGTGCAGTTCATCGTGATC
Xchc2    337 AATCTGGTGGTGGGCCTGGTGGTTTTCTTGATCATCACCACCGTGCAGTTCATCGTGATC

pBS217  7123 GCCAAGGGTTCGGAGCGCGTGGCCGAGGTCGGCGCGCGCTTCACGCTGGACGCCATGCCC
Xchc2    397 GCAAAGGGTTCCGAACGCGTTGCCGAGGTCGGTGCGCGCTTCACGTTGGACGCAATGCCC

pBS217  7063 GGCAAGCAGATGAGCATCGACGCCGACCTGCGCGCCGGCCACCTGTCGCCCGAAGAGGCC
Xchc2    457 GGCAAGCAGATGAGTATCGATGCCGACCTGCGTGGCGGCAACCTGACCGCAGATGAGGCA

pBS217  7003 CGCAAGCGCCGCGCGCTGCTGGCGATGGAGAGCCAGCTGCACGGCGGCATGGACGGCGCC
Xchc2    517 CGCCGCAAACGCGCGCGGCTGGCCATGGAAAGCCAACTGCACGGCGGCATGGATGGCGCC

pBS217  6943 ATGAAGTTCGTCAAGGGCGACGCCATCGCCGGCCTGGTCATCACGCTGGTCAACATCCTG
Xchc2    577 ATGAAATTCGTCAAGGGTGATGCGATCGCCGGCCTGGTGATTACCATGGTCAACATCCTG

pBS217  6883 GCCGGCATCGTGATCGGCATCACGTACCACAACATGACCGCCGGCGAGGCGGCCAACCGC
Xchc2    637 GCCGGTATCGTGGTCGGCGTGACCTACCACGGCATGACGGCCGGCGATGCCGCCAACCGC

pBS217  6823 TTCGCGGTGCTGTCGATCGGCGACGCCATGGTCTCGCAGATCCCGTCGCTGCTGATCTCG
Xchc2    697 TTTGCGATCCTGTCGGTGGGCGATGCGATGGTGTCGCAGATCGCCTCACTGCTGATATCG

pBS217  6763 GTGGCGGCCGGCGTGATGATCACCCGCGTCTCCGACGAAGAGCAGGCC 6716
Xchc2    757 GTGGCCGCCGGCGTCATGATCACCCGCGTGGCCAACGAGAACGAGACC 804

 Score = 908 (250.9 bits), Expect = 5.7e-286, Sum P(3) = 5.7e-286
 Identities = 276/394 (70%), Positives = 276/394 (70%), Strand = Minus /
Plus

pBS217  6030 GTGCGCACCTGTTCGTCGGCCTGCAGGAAACGCAGTGGATGCTCGAGCGCGTCACCACCG
Xchc2   1352 GAGCGCACTTGTTCGTCGGCATCCAGGAGACGCAGTGGATGCTGGAGCAGGTGGCCGTGG

pBS217  5970 ACTACCCCGGCCTGGTGGCCGAAGCGCAGAAGGCCGTGCCGGCCCAGCGGATCGCCGACG
Xchc2   1412 ACTACCCCGGGCTGGTTGCGGAGGTCAACAAGGCGATGCCCGCCCAGCGCATAGCCGATG

pBS217  5910 TGCTGCGGCGCCTGCTGGAAGAGCAGGTGCCCATCCGCAACATGCGCGCCATTCTCGAGA
Xchc2   1472 TGTTGCGGCGCCTTCTGGAAGAAAGGATTCCGGTGCGCAATATCAAGAGCATTCTGGAAA
```

```
pBS217  5850 GCCTGGTGGTCTGGGGCCCGAAGGAGAAGGACACGCTGATGCTGGTGGAATACGTGCGCG
Xchc2   1532 GTCTGGTGGTCTGGGGGCCGAAGGAAAAGGATCTTCTGATGTTGACCGAGTATGTGCGCT

pBS217  5790 GCGACCTGGGACGGCAGATCGCGCACCAGGCCACCGGCGGCACCCGCCAGATGCCGGCCA
Xchc2   1592 GCGATCTGGGCCGTTATCTGGCGCATACCGCAACCGCAGGCACCGGGCAGCTGCCCGCAG

pBS217  5730 TCCTGCTGGACCTGTCGGTCGAGCAGACCGTGCGCCAGGCCATCAAGCCGACGCCGGCGG
Xchc2   1652 TGATGCTCGACCATGCTGTCGAACAACTGATCAGGCAATCGATTCGCGCGACCGCGGCCG

pBS217  5670 GCAACTTCCTGACGCTCGATCCGCAGCAGGTGGA 5637
Xchc2   1712 GCAATTTCCTGGCACTTCCGCCCGAGCAGGCGAA 1745

 Score = 318 (87.9 bits), Expect = 5.7e-286, Sum P(3) = 5.7e-286
 Identities = 98/141 (69%), Positives = 98/141 (69%), Strand = Minus /
Plus

pBS217  5577 CGCTGGCCATCGTGACCTCGATGGACATCCGCCGCTATGTCCGGCGCATGATCGAGCCGC
Xchc2   1796 CGCTGGCGGTGGTCGCGTCGATGGACGTGCGCAGGTATGTGCGCCGCATGATCGAAGCAC

pBS217  5517 ACCTGCAGGCGCTGAACGTGTATTCGTTCCAGGAGCTTGGCGGCTACGTCGACCTGCGCC
Xchc2   1856 GGCTGACCTGGCTGCAGGTCTATTCGTTCCAGGAGCTGGGTTCGGAGGTGCAGCTGCAGC

pBS217  5457 CGGTCGGCAAGCTGGTGCTGT 5437
Xchc2   1916 CGATCGGTAGAGTGGTGGTGT 1936
```

Appendix IV

Potential internal priming sites for *hrpX* primer sets

No significant sites were identified - Upper case shows region of highest similarity

```
HrpXc        gatatcaatactcaaccggatggcgcgaaatgtctggcgtacgacaatgccaacgatgtg   60
HrpXo        ------------------------------------------------------------    0

HrpXc        caaatgcgctttgcgtcgggagaaaacgcatcacatggttgtttgacagagtgagctttt  120
HrpXo        ------------------------------------------------------------    0

HrpXc        ttgatttttcaatggctttcagagggaaattctattGGATCCGCTGCATACAATCGTTT   180
HrpXo        -------------------------------------GGATCCGCTGCATACAATCGTGT    23

HrpXc        GCGCCAGCGAGTTCGGCGCCTGTTGTCTTTTGCTCCGCCCCCCAAaGAGAGAGACCGGCA   240
HrpXo        GCGCCACGGAGCTCGGCGATTGTTGTCTTTTGCTCCGCCCCCCAA-GAGAGAGACCGGCA   82

HrpXc        TCATCCTTTCCACCTACTTTGCAGCGATCTCTGCGTTGTCATACGCAGAACGTCTTCCTA   300
HrpXo        TCATCCTTTCGACCTACTTTGCAGCGATCTCTGCGTTGTCTTACGCAGAACGTCTTCCTA   142

HrpXc        CCTATACGAGCAGGATGCTGGTTGGTGCTTGGCCACAGGGACTGCAACATCTCCAACAGC   360
HrpXo        CCTATACGAGCAGGATGCTGGTTGGTGCTTGGCCGCAGGGACTGCAACACCTTCAACAGC   202

HrpXc        GACGCGACGGACAGGGCGCAGCGGATGGCGCCGACGATGAGGTCAGCTTGTTCGGTGCCA   420
HrpXo        GACGCGACGGGCAGGGTGCAGCCGATGGCGCCGACGATGAGGTCAGCCTGTTTGGTGCCA   262

                                      AGTGGCCCGCGCGCGACGA
HrpXc        GCGGCGATGCGTTGCTGATCCTGGAATATCAGGAAGAGGCCGAAGATGCGTATCGGCAGG   480
HrpXo        GCGGCGATGCGCTGCTGATCCTGGAATATCAAGAAGAGGCCGAAGACGCGTATCGGCAGG   322
                POTENTIAL REVERSE PRIMER SITE - 229/209bp PRODUCT/S
                     AGTGCCCCCCGCGCGACGA
HrpXc        CCTTGAAGGCCATGCGCGGGCATCAGCGCCAATTGCGCCTGCTGTCGTGCCGGAACACCG   540
HrpXo        CTTTGAAAGCCACTGCGCGGGCAGCAGCGCCAATTGCGTTTGCTGTCGTGCCGGAATACC   382
                POTENTIAL REVERSE PRIMER SITE - 293/273bp PRODUCT/S
                           AGTGGCCCGCGCGCGACGA
HrpXc        CCTGGTTGATGCTGAGACAGCGGCGCCTGAGCGCGGCGTTGAATTGCTTCGCGCAGTTGG   600
HrpXo        GCCTGGTTGATGCTGAGCCAGCGACGCCTGAGTGCGGCATTGAACTGCTTTCGCAGCTGC   442
                POTENTIAL REVERSE PRIMER SITE - 338/308bp PRODUCT/S
                                                                     AGT
HrpXc        CAATGGACCGCGAAACGCCGCCCAGCCTGTGCGGCGAGAGCCTGTTGGGCAAATCGCTCA   660
HrpXo        GATGGATCGCGAAACGCCCCCCAGCCTGTGCGGCGAGAGCCTGCTGGGCTCAAGGCACTG   502

             GGCCCGCGCGCGACGA
HrpXc        CCCACTTCCATCTGGGCCAGAGCACGTTGGCCTTACAGACCCTGGAGCGTGCCCGCGAGG   720
HrpXo        ACCCACTTTCATCTGGGCCAGAGCACGCTGGCCTTGCAGACCCTGGAGCGCGCCAACGAG   562
                POTENTIAL REVERSE PRIMER SITE - 438/458 bp PRODUCT/S

HrpXc        TGCTGGCCGACCTGGAGAGCGGCGCGTCGGACTGGCTGCGGATTGCCACTGCGCTGCGTC   780
HrpXo        GTGCTGGCCGAGCTGGAGAGTGGTGCTTCCGATTGGCTGCGTCTCGCCACCGCGCTGCGG   622

              AGTGGCCCGCGCGCGACGA
HrpXc        TGGACATGATTGCGCAGCTGCGTATCCGTCGCTCCGACCGCATGAGCGACCATGTGTTCT   840
HrpXo        CTGGACATGATCGCGCACTGCGCATCCGCCGGTCCGATCGCATGGTCGACCACGTGTTCT   682
                POTENTIAL REVERSE PRIMER SITE - 540/560bp PRODUCT/S

HrpXc        GGCAAGCCACGCTGCGCCAGGAAGATGCCACGCATGCCTTCGAGCCCAGCGACCTGCGCG   900
HrpXo        GGCAGGCCACGCTGCGCCAGGAAGATGCCGCGCATGCCTTCGAGCCCAGCGATTTACGCG   742
```

```
HrpXc       CCTGCATCGCCGATCTGGCAGAGAGCATGCCGGTGCTTGCGCAGCGCATGCGGCATGTAC   960
HrpXo       CATGCATCGCCGATCTGGCCGAGAGCATGCCGGTGCTGGCGCAGCGCATGCGGCATGTGC   802

                        POTENTIAL FORWARD PRIMER SITE - 485bp PRODUCT
HrpXc       GCAGCCTGTTGCGTATTGCCGGCGGCGACACCTTCGGCTTCGACGCGCAGATCGACGCAT  1020
HrpXo       GCAACCTGCTGCGGATCGTCGGCGGCGATACCTTCGGCTTCGATGCGCAGATCGATGCGT   862

HrpXc       TGCCGGCCGCAGCCACCGGCTGCCCGCCGTGTGCGCGTCAGGACGCGCAGGTGGAACTGG  1080
HrpXo       TGCCGGCTGCGGCCACCGGCTGCCCGCCGTGTGCGCGTCAGGACGCACAGGTGGAACTGG   922

HrpXc       CGCTGGCGGCATTGGCGGTGGGCCGTGCCGATCTGGCCGAGCGTGCGCTGGCCGGTGCCG  1140
HrpXo       CGCTTGCCGCATTGGCGGTGGGGCGTGCCGATCTGGCCGAGCGCGCGCTGGCCGGTGCCG   982

HrpXc       GCCGACATCATGCGCCGCGCTGGAACCTGGAGTTCGAGTACTGCCAGGCCAAGATCAGCC  1200
HrpXo       GCCGGCATCATGCGCCGCGCTGGAATCTGGAGTTCGAATACTGCCAGGCCAAGATCAGTC  1042

HrpXc       AGGCGATGGGGCGCACCGAACAAGCGTTGCTGCTCTACAACCGCTACGCGCTGGGCGCGG  1260
HrpXo       AGGCGCTGGGACGCACGGAACAAGCGTTGTTGCTCTACAACCGCTATGCGCTGGACGCGG  1102

                  TACTTTGCAGCGATCTCTGCG
HrpXc       TGCAGTGCCTGCGCAGCGAAGTGCAGGCACCGCGCTTGCTGGAAAGCGGCACCCCGGCGC  1320
HrpXo       TGCAATGCCTGCGTAGCGAAGTGCAGGCACCGCGCTTGCTGGAAAGCGGTACCCCAGCGC  1162
               POTENTIAL FORWARD PRIMER SITE - 185bp PRODUCT

HrpXc       ACGTCAGCGACGATATCTCCGCCCGGTTGCCGGCCAAGTATCGGCGCGCCTACAGCTACA  1380
HrpXo       ACGTCTCCGACGATATTTCCGCCCGCTTGCCGGCCAAATATCGGCGTGCGTATAGCTACA  1222

HrpXc       TGATCACCAATGCGCATCGCTCGGATCTGTCGATCAACGAAGTGGCCGCACAGATCGGGG  1440
HrpXo       TGATCACCAACGCGCACCGCTCGGATCTGTCGATCAATGAAGTGGCCGCACAGATTGGGG  1282

            AGTGGCCCGCGCGCGACGA
HrpXc       TCACCGGGCGCGCGCTgcaGCAAGCGTTCAAGTCGGCTACCGGGCTATCGCCGACGCAGG  1500
HrpXo       TGACCGGGCGCGCACT---GCAGGCGTTCAAGTCGGCCACCGGGCTGTCGCCGACCCAGG  1339

HrpXc       TGCTGCGCCGCTACCGGATGCAAAGCATCCGCGACGAACTGCTGGCCGAAGGcgGCTGCG  1560
HrpXo       TGCTGCGCCGTTACCGCATGCAGAGCATCCGCGACGAACTGCTGGCCGAAGG--GCTGCG  1397

HrpXc       GCAGTGTGTTGCAGGCGGCCAGTCGCTGGGGCGTCGGCAGCCGTTCGGCGTTGGCCAAGG  1620
HrpXo       GCAGCGTGTTGCAAGCGGCCAGCCGCTGGGGTGTGGGCAGCCGTTCGGCATTGGCCAAGG  1457

HrpXc       GCTATCGCCAGCACTTCAATGAGGCGCCGATGGAGACCCTGCAGCGGTAATCTCcgcaac  1680
HrpXo       GCTATCGCCAGCACTTCAACGAAGCACCGATGGAAACCTTGCAACGGTAATCTCttggca  1517

HrpXc       ccacggattggtagcgctaccgatccgtggtgggtcaacccggcgtgcggtgcagtgcgc  1740
HrpXo       cgccacggcttggtagcgctacctgccgtgg----------------------------  1548

HrpXc       gtcgctgtacgcaacggagggcaggggtcaaactgagacgtgcgtgcaatgtgggttccg  1800
HrpXo       ------------------------------------------------------------  1548

HrpXc       gctagc                                                        1806
HrpXo       ------                                                        1548
```

Appendix V

Growth curves for SAM116 and SAM116R in nutrient Broth and M9 minimal medium with 0.5% glucose

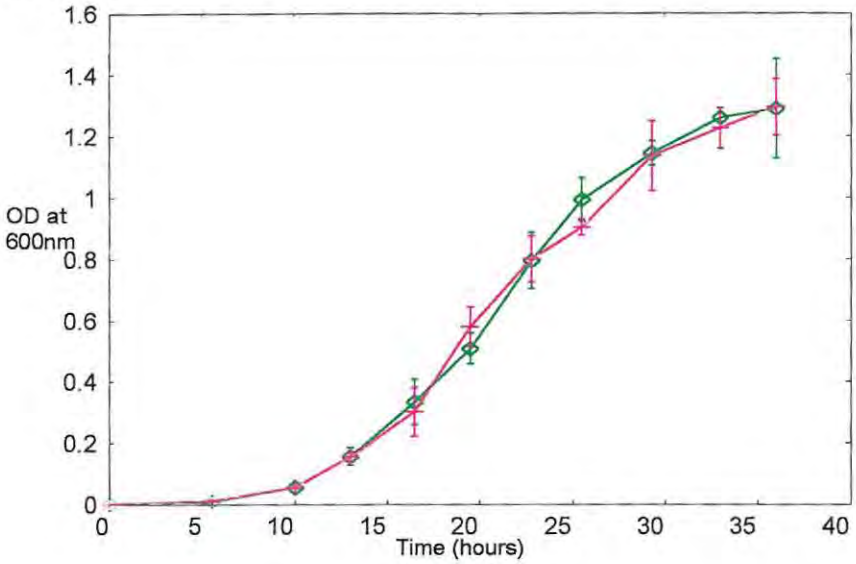

Figure V-1. Growth curves of SAM116 and SAM116R after inoculation of fresh overnight culture of the appropriate organism, at a concentration of 1:50 into 200ml pre-warmed nutrient broth. Incubation was at 28°C and 180 rpm. 1 ml samples were taken periodically and absorbance at 600nm measured. Points represent the average of three samples, and error bars the standard deviation from the mean.

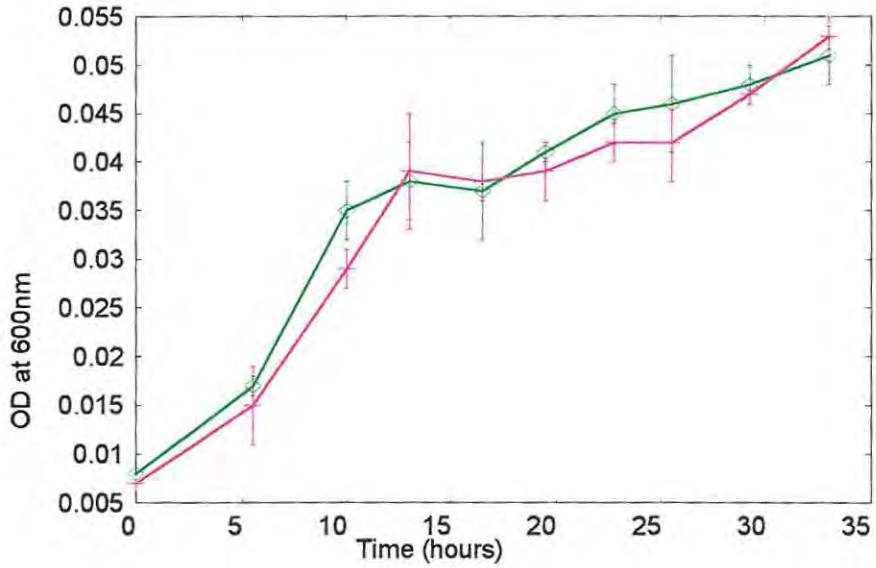

Figure V-1. Growth curves of SAM116 and SAM116R after inoculation of fresh overnight culture of the appropriate organism, at a concentration of 1:50 into 200ml pre-warmed M9 minimal medium. Incubation was at 28°C and 180 rpm. 1 ml samples were taken periodically and absorbance at 600nm measured.

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