Abstract

*Xanthomonas oryzae* pathovar *oryzae* (*Xoo*) is the causal agent of bacterial leaf blight, a serious disease of rice. It is a vascular pathogen, which grows in the xylem vessels and causes drying of infected leaves. Previously, mutations affecting extracellular polysaccharide (EPS) production, regulation of pathogenicity factor (*rpf C*), Hypersensitive Response and Pathogenicity (*Hrp*) genes, certain avirulence (*avr*) genes and a few amino acid biosynthetic genes have been found to be associated with virulence deficiency. The aim of this study is to identify other virulence functions of *Xoo* using genetic and molecular biology techniques.

Chapter 1 is a summary of bacterial virulence and has been divided into three parts. The first part deals with bacterial secretion systems, adhesins and effector molecules and their role in virulence. The second part deals with methodology for studying bacterial virulence functions and in the third part evolution of bacterial virulence with reference to the genomic organisation of virulence genes has been discussed.

Chapter 2 describes the isolation and characterisation of virulence deficient (*Vir−*) mutants of *Xoo*. *Vir−* mutants were isolated by screening Ethyl Methane Sulfonate (EMS) and transposon 5 (Tn5) induced mutagenised cultures of *Xoo*. From the mutagenised cultures, EPS and pigment proficient colonies of *Xoo* were inoculated on rice plants and the strains which exhibited reduced lesions upon repeated inoculations were considered as *Vir−* mutants. These *Vir−* mutants were further characterised for prototrophy, growth on laboratory medium,
motility and Hypersensitive Response on non host plant, tomato. Mutants that were proficient for all these characters but still unable to cause disease were taken for further study because these were likely to define novel virulence functions in \textit{Xoo}. Phytopathogenic bacteria are known to produce extracellular enzymes like cellulases, proteases, pectinases and xylanases which degrade plant cell wall components. We have reported for the first time that \textit{Xoo} secretes a xylanase, which is an enzyme involved in the degradation of xylan, a major component of rice cell walls. One \textit{Vir}^{-} mutant (BX0801) was found to be xylanase deficient (Xyn^{-}) and taken for further study.

Characterisation of BX0801 has been discussed in Chapter 3. A cosmid clone (pSR1) which restores xylanase and virulence proficiency to BX0801 was identified from a genomic library of wild type \textit{Xoo} by functional complementation. pSR1 carries an insert DNA of 29.7 kb which consists of 11.0, 9.0, 6.2 and 3.5 kb \textit{EcoRI} fragments. By restriction digestion analysis and Southern hybridisation it was confirmed that the organisation of the \textit{EcoRI} fragments in cloned DNA in pSR1 is same as in the \textit{Xoo} genome. Transposon5 and Transposon10 were used to mutagenise pSR1 and the insertions obtained were marker exchanged into the genome of the wild type \textit{Xoo} strain. The marker exchange mutants obtained were classified as class I (\textit{Vir}^{-}, Xyn^{-}), class II (\textit{Vir}^{-}, Xyn^{+}) and class III (\textit{Vir}^{+}, Xyn^{+}). Sequence analysis using transposon specific primers revealed that the genes required for virulence and xylanase proficiency are homologues of \textit{xpsF} and \textit{xpsQ} which encode components of a type II protein secretion system in \textit{Xanthomonas campestris} \textit{pv. campestris}. Assay of xylanase activity in various cellular compartments showed xylanase accumulation in
cytoplasm and the periplasmic space of the xpsF mutant. The clone pSR1 restores transport of xylanase to the extracellular space in this mutant. SDS-PAGE analysis of extracellular proteins showed that in addition to xylanase, secretion of certain other proteins was also affected in xpsF mutant of Xoo. It is known that Xoo Hrp- mutants that are deficient in a different (Type III) protein secretion system can be complemented for growth in planta by coinoculating with the wildtype strain. Coinoculation studies with wild type Xoo strain showed that xpsF mutants could not be complemented for growth in planta. This suggested that in addition to cell independent functions, type II protein secretion system also functions in cell dependent manner.

The characterisation of one of the class II (Vir-, Xyn+) mutants is discussed in Chapter 4. In this mutant (BXO808), a Tn5 insertion (vir-2::Tn5) is located in a genomic region that is part of the 9.0 kb EcoRI fragment cloned in pSR1. Besides a virulence deficiency, BXO808 also exhibits an altered lesion phenotype. The lesions caused by this mutant are localised mainly to the side parallel veins whereas those caused by the wild type strain extended primarily through the mid-veinal region. The pSR1 clone as well as a subclone (pSR3) carrying the 9.0 kb EcoRI fragment restore virulence and normal lesion phenotype to the BXO808 mutant. Sequence analysis indicated that the vir2::Tn5 insertion is present in an ORF of 3795 nucleotides which potentially encodes a protein of 1265 amino acids. The encoded protein exhibits homology to adhesins from several animal pathogenic bacteria like YadA protein of Yersinia enterocolitica, UspA1 protein of Moraxella catarrhalis and antibody binding protein (elb) of certain pathogenic Escherichia coli strains. Protein sequence analysis using appropriate software also
suggests that this protein belongs to a family of autotransporters present in the outer membrane of Gram-ve bacteria. Several other transposon5 insertions were obtained in this ORF that eliminate the complementing activity for virulence as well as the lesion phenotype. This confirms that the Tn5 insertion in the 3.795 kb ORF contributes to loss of virulence of the BX0808 mutant. The other Tn5 insertions in the 3.795 kb ORF were also marker exchanged into the Xoo genome. Surprisingly, none of the mutants exhibited either a virulence deficiency or an altered lesion phenotype. It is quite unusual that a transposon insertion in a gene exhibits a particular phenotype while other insertions (both upstream and downstream of the insertion site) in the same gene have no phenotype. We are now examining the possibility that the phenotype of the BX0808 strain is due to the vir-2::Tn5 insertion and a second mutation elsewhere in the genome.

In conclusion, we have identified an Xoo gene that encodes a putative outer membrane protein that exhibits homology to adhesins of animal pathogenic bacteria. This gene may be contributing to virulence of Xoo in a tissue specific manner.