Synopsis

*Xanthomonas oryzae* pv. *oryzae* (Xoo) is a plant pathogenic, Gram-negative bacterium which causes bacterial leaf blight (BLB) of rice. The disease can result in yield losses of up to about 50% in the major rice growing regions of Asia and tropical Africa. Xoo is about 2μm in length, rod shaped with a single polar flagellum. In natural conditions, Xoo enters inside the rice leaf primarily through hydathodes which are specialized water pores located in leaf tips and margins and then migrates through the xylem vessels and causes the death of the leaves leading to the development of characteristic disease lesions. In BLB disease development, Xoo adhesion to rice leaves is a very initial step in the encounter between the host and bacterium. Bacterial adhesion to host surface is a crucial and fundamental step in pathogenesis. Molecular mechanisms of the functioning of several virulence factors of plant pathogenic bacteria are known today but very little attention has been given to study the adhesin function of plant pathogenic bacteria. A previous study in our lab has shown that *Xanthomonas* adhesin-like protein A (XadA) exhibits high sequence homology with adhesins of animal pathogenic bacteria and is required by Xoo for optimum virulence in the natural mode of infection (i.e., hydathodal entry). This was one of the first reports on the role of an adhesin-like protein in virulence of any plant pathogenic bacterium.

The present study has three main objectives:

1. Identification of the genes for adhesin-like functions in the Xoo genome by bioinformatics/*in silico* analysis and generation of mutations in the selected genes.
2. Development of assays to study the initial stages of leaf colonization/entry by Xoo.
3. The application of these assays as well as tests for virulence in order to understand the contribution of these adhesin-like functions in early stages of leaf colonization/entry and virulence of Xoo.

The first chapter is the introductory chapter of the thesis. In this chapter, I begin with an introduction to bacterial diseases of plants and to Xanthomonas oryzae pv. oryzae (Xoo), the subject of study in this thesis. A brief overview of virulence factors of pathogenic bacteria is presented. I then provide a more exhaustive account of the different kinds of adhesins that are known to be employed by animal and plant pathogenic bacteria. Bacterial adhesins are proteins which are localized on the bacterial membrane and dock on the cognate receptors present on the host cell surface. All the adhesins are categorized into two major groups: afimbrial and fimbrial adhesins. While afimbrial adhesins are single proteins, fimbrial adhesins are made of several protein subunits. A brief note on the theoretical approach of bacterial adhesion has been also presented. A discussion of various avenues for entry of plant pathogenic bacteria inside the host plants has been also included. Different experimental approaches to address the bacterial adhesion process have been also mentioned. I end the introductory chapter by providing the information that is currently available about the adhesion/entry functions of Xoo.

The second chapter deals with an in silico approach to identify the adhesin-like molecules that are encoded in the Xoo genome and the generation of mutations in the genes for these candidate adhesins. The genome sequences of two Xoo strains are available till date: Xoo KACC10331 and Xoo MAFF311018. The first adhesin-like molecule that I have identified is Xanthomonas adhesin-like protein B, designated as XadB (X000681) in the present work. It is a paralog of previously identified XadA protein. There is about 40% identity in the protein sequence of these two paralogs. Both XadA and XadB exhibit high sequence homology with adhesins of animal pathogenic bacteria. I have also found one ortholog of the Yersinia virulence factor, called YapH (Yersinia autotransporter protein which is predicted to be
an adhesin), in the Xoo genome. The Xoo homolog of YapH (XOO2380) is predicted to be the second largest protein in the Xoo genome and has several sequence features which suggest that it might be an adhesin. All these three proteins: XadA, XadB and YapH, belong to the afimbrial group of adhesins. Xoo genome also harbours a set of genes predicted to encode Type IV pilus, a well known virulence as well as adhesin factor in several animal pathogenic bacteria. PilQ protein forms the outer membrane located secretin through which pilin subunits assemble to form the rod like Type IV pilus structure. Mutation in the pilQ gene has been reported to result in an inability to form the Type IV pilus and the loss of associated functions in many animal pathogenic bacteria. For my study in this work, I selected the pilQ gene. At the initial stage of my work, I confirmed the presence of all these three genes (i.e. xadb, yapH and pilQ) by PCR amplification and sequencing in the genome of BX043, the lab strain used as the wild type in my study. For generation of mutations in xadb, yapH and pilQ genes, I followed the gene disruption method. An internal fragment of the candidate gene was PCR amplified and cloned in pK18mob vector. This recombinant plasmid was then mobilized into the Xoo cells and single recombinants obtained by homologous recombination were selected. These mutants were confirmed by PCR using different pairs of gene specific and vector specific primers. The orientation of the insert was selected in such a way that it leads to the generation of non-polar mutations (i.e. those that do not affect transcription of genes that are downstream of the gene of interest).

The third chapter describes the development of an assay for studying the early stages of leaf colonization and entry by Xoo and the use of this assay for understanding the role of the XadA, XadB, YapH and PilQ proteins in this process. The assay is based on the use of Enhanced Green Fluorescent Protein (EGFP) tagged Xoo cells and the confocal laser scanning microscope. The results presented in this chapter indicate that Xoo cells are capable of adherence and entry into rice leaves within one hour of infection. Mutations in xadA, xadB and yapH affected the processes of leaf colonization and entry while the pilQ mutation did not appear to affect these processes. The
experimental data clearly provide the evidence that XadA, XadB and YapH play a role in promoting optimal leaf adhesion and hydathodal entry. I have also used scanning electron microscopy (SEM) to study the effect of mutations in these adhesin-like functions on the ability of Xoo to associate with hydathodal surfaces. The results indicate that the XadA protein plays an important role in promoting colonization of the surface of hydathodes by Xoo while the XadB, YapH and PilQ (and hence type IV pilus) proteins may have minor roles in influencing this process. Therefore, all the experiments carried out in this chapter suggest that certain afimbrial adhesin-like functions are indeed important for optimum adhesion and entry into the rice leaves.

The fourth chapter describes the virulence properties of the different Xoo mutants that were generated in this study and also includes an examination of their \textit{in vitro} adhesion and autoaggregation phenotypes. Two different assays were used to assess the virulence property of different Xoo strains. The first assay, which is more akin to the natural mode of infection, requires entry into the rice leaves through the hydathodes (which are water pores located at the tip and margins of rice leaves). This mode of infection is called the epiphytic mode of infection as the bacterium is deposited on the leaf surface and subsequently has to make its way into the plant. The second method of infection involves wound inoculation which is performed by clipping the leaf tips with scissors dipped in bacterial inoculum. This method of infection obviates the need for entry through the hydathodes and allows us to assess the role of specific adhesin-like functions in the post-entry phase; i.e. during growth within the xylem vessels. Following epiphytic mode of infection, the \textit{xadA}, \textit{xadB} and \textit{yapH} mutants exhibited virulence deficiency. However, the \textit{pilQ} mutant showed some variability as this mutant was found to exhibit a virulence deficiency in only 50% (2/4) of the experiments. The data indicate that the afimbrial proteins (XadA, XadB and YapH) are required for optimum virulence following epiphytic infection. However, in the wound inoculation method, the effect on virulence was very different. The \textit{pilQ} mutant was the most virulence deficient amongst the four strains analyzed. The \textit{yapH} mutant had a moderate virulence deficiency. There was no virulence deficiency
associated with either the xadA\(^{-}\) or xadB\(^{-}\) mutant following wound inoculation. I also examined the effect of these different mutations on the ability of the bacterium to migrate within rice leaves following infection by wound inoculation. The pilQ\(^{-}\) mutant exhibits a severe deficiency in its ability to migrate within the rice leaves. The yapH\(^{-}\) mutant exhibits a moderate deficiency in the ability to migrate within the rice leaves. The xadA\(^{-}\) and xadB\(^{-}\) mutants migrate through rice leaves as well as the wild type strain. It appears that PilQ protein (and hence Type IV pilus) is an important virulence factor that promotes growth/migration of Xoo within the xylem vessels. The YapH protein also promotes growth/migration within the xylem vessels. The XadA and XadB proteins are not required for Xoo growth/migration in the xylem vessels. Defects in in vitro adhesion property, cell-cell interactions and microcolony formation have been reported to be associated with mutations in the genes for adhesin-like molecules of diverse microbial cells. In the present study, no difference was found as compared to the wild type strain, in the ability of any mutants to attach to a glass surface. However, when the autoaggregation property of the Xoo mutants was observed, it was found that the XadA, XadB and PilQ proteins appear to play a role in cell-cell interactions as well as in the formation of small bacterial clusters.

Overall, the experiments carried out in the present work indicate that XadA, XadB, YapH and PilQ/Type IV pilus promote optimum virulence of Xoo. The XadA protein, and its paralog XadB, appear to be important for promoting the early stages of leaf colonization and entry. The YapH protein appears to promote early stages of leaf colonization and entry and also appears to play a role in promoting lesion formation/migration during growth within rice xylem vessels. The type IV pilus plays a major role in promoting lesion formation/migration during growth within the rice xylem vessels. The results obtained in this study indicate that Xoo adheres to rice leaf surfaces by means of several afimbrial adhesin-like proteins, of which XadA plays the most important role, followed by XadB and YapH proteins. Entry inside the plants through the natural openings like hydathodes is also carried out by the functions of the same proteins. Adhesion to the surface of hydathodes is
primarily dependent on XadA although XadB, YapH and Type IV pilus may play a minor role. Once inside the xylem vessels, the PilQ protein/Type IV pilus is required for optimum virulence although the YapH protein also has a role at this stage. The results also suggest that multiple adhesin-like functions are involved, to different extents, in promoting the various stages in the process by which Xoo infects rice plants.