Chapter one
Introduction

1.1 Plant pathology and bacterial diseases

Plant diseases are caused by several pathogenic agents like fungi, insects, bacteria and viruses. Different diseases of crop plants cause an enormous yield loss every year throughout the globe. Also, certain diseases of plants that were earlier considered to be minor (in terms of degree of yield losses) are becoming more severe and some of these may be due to increased temperature as a result of global warming. The overall global situation is such that human population is increasing while the amount of arable land is decreasing (due to a variety of reasons). Therefore, it is important to safeguard, against losses due to plant diseases, as much as possible of what has been produced. In this context, understanding the mechanisms by which plant pathogens cause diseases becomes very important. This knowledge might be the key towards developing new strategies for protecting crop plants against their pathogens.

The first microscopic detection of bacteria was achieved in the seventeenth century when Antonie van Leeuwenhoek described some oral bacteria. Almost two hundred years later, bacteria were found to be agents of plant diseases when Burril (1878) first reported bacterial association with fireblight of apples and pears (Burrill, 1878). Since then, a number of important plant diseases have been reported to be caused by bacteria of diverse taxonomic groups.

Plant disease development involves several discrete but successive steps, such as, inoculation, adhesion, entry, invasion, multiplication and dissemination of the pathogen. Inoculation which marks the contact between a pathogen with its host is the beginning of the infection process. Plants also exhibit several novel mechanisms to resist the bacterial invasion at different
stages of infection. The technological innovations and vast amounts of information developed through genome sequencing programs provide a great platform to understand the molecular basis of plant-pathogen interactions.

1.2 *Xanthomonas oryzae pv. oryzae* (Xoo)

The genus *Xanthomonas* belongs to subdivision gamma-proteobacteria that causes diseases on at least 124 monocotyledonous and 268 dicotyledonous plant species (Chan et al., 1999). One of the very important members of this group is *Xanthomonas oryzae pv. oryzae* (Xoo). The bacterium causes a serious vascular disease of rice called bacterial leaf blight (BLB) which is prevalent in the rice growing areas of Asia and parts of West Africa. The maximum yield loss may be as high as 50% depending on the rice variety, the growth stage of the plant at the time of infection and climatic conditions (Elings et al., 1997). Leaf blight of rice was first discovered in Japan in 1884 but acidic soil was attributed as a causal agent. Later in 1909, bacteria were found to be associated with this disease (as reported by Nino-Liu et al., 2006). Later, the causal bacterium was isolated and named as *Bacillus oryzae* (Bokura, 1911, as mentioned by Mizukami et al., 1969). The bacterium got different names in subsequent years as *Psedomonas oryzae*, *Xanthomonas oryzae* (Ishiyama, 1922) and *Xanthomonas campestris pv. oryzae* (Dye, 1978). In 1990, the pathogen finally got the present name and status as a species, *Xanthomonas oryzae pv. oryzae* (Goto, 1992; Swings et al., 1990) in the family Xanthomonadaceae.

Xoo is a rod shaped, round ended, capsulated, Gram-negative, motile species with a single polar flagellum, non-spore former, obligately aerobic and is infected with filamentous bacteriophages (Nino-Liu et al., 2006). The optimum temperature for growth is 25-30°C. A single cell measures in length from approximately 0.7-2.0 μm and in width from 0.4-0.7 μm (Nino-Liu et al., 2006). Colonies on solid media are round, convex, mucoid and yellowish in colour due to the production of the pigment xanthomonadin, characteristic of the genus (Bradburry, 1984).
1.3 Bacterial virulence factors

Plant pathogenic bacteria employ several strategies for a successful infection of the host plant. Higher plants contain potentially vast sources of nutrients for the bacterial species and bacteria, owing to small sizes, can enter inside the plant tissues through natural openings like stomata, hydathodes etc. which are present on the plant surfaces. However, only a fraction of the bacterial species manages to survive in such an environment because bacterial survival inside plant tissues requires specialized machinery to establish a metabolic relationship with the host. Research conducted during the last few years, in a number of laboratories worldwide, has led to the discovery of a number of virulence factors of plant pathogenic bacteria. In the next section, I provide a very brief overview of some of these virulence factors, with particular reference to those that have been shown to be absolutely essential for pathogenicity of a number of plant pathogenic bacteria as well as those that are known to be required for virulence of Xoo, which is the subject of this study.

1.3.1 The HRP system (Hypersensitivity response and pathogenicity)

The \textit{hrp} genes are essential for pathogenicity of a number of Gram-negative bacterial plant pathogens (including Xoo) and are also involved in elicitation of hypersensitive response (HR; a form of plant defense response) in the non-host plants (Puhler et al., 2004). The Hrp system includes a protein secretion system (called the type III secretion system or TIIISS) which is described in a later section in this chapter that is specialized for secreting proteins directly into the cells of the eukaryotic hosts. Some of the \textit{hrp} genes also encode for the proteins that are secreted through the TIIISS as well as regulatory proteins that coordinate the expression of these proteins (Lindgren, 1997).

1.3.2 Bacterial Toxins

Several pathogenic bacterial species secrete various toxins which are mostly small peptides and highly diffusible secondary metabolites. Often these molecules serve as important virulence factors. The specific role of individual
toxins in pathogenesis is not very clear. *Pseudomonas tabaci*, the causal agent of wildfire disease of tobacco, produces a chlorotic halo due to the secretion of a toxin which affects host methionine metabolism and this toxin may even kill young plants (Braun, 1950). *Xanthomonas albilineans*, which causes leaf scald disease of sugarcane, produces a highly potent pathotoxin called albicidin (a polyketide-peptide compound) which has been shown to be a potent inhibitor of supercoiling activity of both bacterial and plant DNA gyrases (Hashimi et al., 2007).

1.3.3 Extracellular polysaccharides (EPS)
Many plant pathogenic bacteria produce EPS as loose slime or as capsular material. These molecules may provide free-living bacteria some selective advantages in stressful environments and are suggested to have several functions such as, adhesion, nutrient acquisition, maintaining water balance, protection against antimicrobial compounds etc. In many plant pathogenic bacteria such as, *Xanthomonas oryzae* pv. *oryzae*, *Ralstonia solanacearum*, *Erwinia amylovora*, EPS has been shown to promote pathogenesis (Denny, 1995).

1.3.4 Lipopolysaccharide (LPS)
Gram-negative bacteria have two membranes- an inner membrane and an outer membrane separated by a periplasmic space. LPS is an important component of the outer leaflet of the outer membrane. The lipid portion of LPS, LipidA, is embedded in the outer membrane while the polysaccharide part is exposed on the bacterial outer membrane. The polysaccharide part consists of an inner core, outer core and an O-polysaccharide, also known as O-antigen. Various studies indicate that LPS has a dual role in plant-microbe interactions (Dow et al., 2000). It may act as an inducer of plant defense responses and may also promote bacterial virulence. LPS may act as a barrier against the anti-microbial compounds secreted by the host, thus promoting survival of the pathogen inside the host.
1.3.5 Flagellar apparatus

Flagella are filamentous structures attached to the bacterial cell surface and help in swimming motility which is considered to be one of the important factors for phytopathogenicity in several bacterial genera (*Pseudomonas*, *Ralstonia*, *Erwinia* etc). For example, mutations in *fliC* and *fliD* genes in *Pseudomonas syringae* pv. tabaci abolish bacterial motility and also cause reduced virulence on tobacco (Ichinose et al., 2003). In Xoo, mutation in *flhF* gene which affects flagellar synthesis causes reduced motility but not virulence (Shen et al., 2001). In a yeast two hybrid screen, FlhF was found to interact with itself, Xa21 (a host protein involved in resistance) and also with the Xoo homolog of *Pseudomonas aeruginosa* PilL protein (involved in type IV fimbrial biogenesis). The significance of the interaction between a Xoo flagellar protein and a rice resistance protein is not known.

1.3.6 Pigments

Members of the *Xanthomonad* group of bacteria produce yellow, brominated, outer membrane located aryl polyene pigments called xanthomonadins. A cluster of genes (*pigA* to *pigG*) is required for pigment biosynthesis. Mutations in these genes affected pigmentation, bacterial survival on leaf surface and showed reduced virulence under natural mode of infection (i.e., epiphytic infection) in *Xanthomonas campestris* pv. campestris (Xcc) as reported by Poplawasky and coworkers (Poplawasky et al., 2000). In Xoo, a mutation in the *aroE* gene (which encodes shikimate dehydrogenase) has been reported to exhibit a deficiency for virulence and pigment production as well as results in aromatic amino acid auxotrophy (Goel et al., 2001). It appears that the virulence deficiency is due to the aromatic amino acid auxotrophy.

1.3.7 Iron, Zinc and phosphate metabolism

Iron is essential for growth and also for virulence of many plant pathogenic bacteria. In many Xanthomonads, Ferric uptake regulator (Fur) protein regulates iron uptake functions. Mutations in the *fur* gene of Xoo resulted in constitutive production of siderophores which are low molecular weight (500-1000 Daltons) iron chelators that bind to Fe$^{3+}$ with high affinity. Mutations in
the fur gene exhibit reduced virulence of Xoo (Subramoni et al., 2005). Similarly, in Xoo and Xcc, mutations in Zinc uptake regulator (zur) exhibit reduced virulence (Yang et al., 2007; Tang et al., 2005). Phytic acid is a major storage form of phosphate in cereals and legumes and phytases are enzymes involved in phytic acid degradation. In Xoo, mutation in phyA (putative phytase A) gene has been reported to cause virulence deficiency in rice (Chatterjee et al., 2003). The virulence deficiency appears to be due to an inability to use host phytic acid as a source of phosphate during in planta growth.

1.3.8 Bacterial protein secretion systems
Bacteria transport a number of proteins across the cell wall. Secreted proteins are involved in a variety of functions, such as, biogenesis of the cell envelope, acquisition of nutrients, motility, adhesion, quorum sensing, virulence etc. The general secretory pathway (GSP) secretes proteins that have particular signal sequences and is found in bacteria, archaea and eukaryotic organelles. While this transport system is sufficient for the secretion of proteins in the Gram-positive bacteria, the Gram-negative bacterial species need protein transport across a second membrane system, the outer membrane (OM). They accomplish this task using a variety of protein secretion systems that have been classified as type I to type VI. A review by Gerlach and Hansel (Gerlach et al., 2007) summarizes several novel mechanisms adopted by pathogenic bacteria for protein secretion.

Protein secretion systems in the Gram-negative bacteria can be broadly subdivided into two major groups: Sec-independent pathways and Sec-dependent pathways. Sec-independent pathways are again sub-grouped into four categories - type I, type III, type IV and type VI secretion system. On the other hand, Sec-dependant pathways (also known as GSP) has three terminal branches for further transport of Sec-transported periplasmic intermediates, which are designated as: chaperon usher (CU) pathway, type II secretion system and type V secretion system. Brief descriptions about these pathways are as follows:
1.3.8.1 The general secretory pathway (GSP)

In GSP, a heteromeric protein complex mediates the transport of unfolded proteins into the periplasm. The bacterial GSP contains two inner membrane located heterotrimeric complexes (SecYEG and SecDFYajC) and an accessory component, SecA (Yahr et al., 2000; Rusch et al., 2007). Proteins with an N-terminal signal peptide are translated as pre-proteins. ATP hydrolysis catalyzed by SecA drives the translocation of the pre-protein into the periplasm. The SecDFYajC complex is likely to enhance translocation through SecYEG by increasing membrane cycling of SecA (Duong et al., 1997). Upon translocation, the signal peptide is cleaved off the pre-protein by specific periplasmic signal peptidases and the mature protein is released into the periplasmic space. Additional signals, if present, might be recognized by the components of the terminal branches (pathways) for subsequent translocation across or insertion into the outer membrane.

1.3.8.2 Type I Secretion System (TISS)

Type I secretion system (TISS), also known as ATP-binding cassette (ABC) transporters are heterotrimeric complexes with the following components: an inner membrane ABC exporter, a membrane fusion protein (MFP) and an outer-membrane protein (OMP). Varieties of substrates are secreted by TISS, from the cytoplasm to the extracellular space without a stable periplasmic intermediate. Most protein substrates of TISS contain a C-terminal signal sequence (Stanely et al., 1991). The mechanism of type I secretion system was studied in great detail using α-hemolysin (HlyA) secretion by certain uropathogenic Escherichia coli strains (UPECs) (Thanabalu et al., 1998). Mutation in a TISS of Xoo has been reported to affect the secretion of a quorum sensing molecule which regulates bacterial virulence associated functions and is also recognized as an avirulence factor by rice cultivars that contain a particular resistance gene called Xa21 (Lee et al., 2006).

1.3.8.3 Type II Secretion System (TIISS)

Type II secretion system (TIISS) is functionally associated with the Sec machinery. Several Gram-negative bacteria use TIISS to secrete enzymes
and toxins across the outer membrane. It's the main terminal branch of the GSP, and the prototype is the secretion system that is used to secrete pullulanase (PulA) by Klebsiella oxytoca (Pugsley et al., 1997). Cryo-electron microscopy shows that PulD forms a ring shaped complex with a central cavity in the outer membrane (OM). The small lipoprotein PulS is required for PulD targeting and insertion (Nouwen et al., 1999). Type II secreton consists of a multi-subunit complex inserted into the inner membrane. In Xoo, it has been shown that several plant cell wall hydrolyzing enzymes like xylanase, cellulase, cellobiosidase, and lipase/esterase are secreted by TIISS and mutations in this system resulted in a virulence deficiency (Ray et al., 2000; Rajeswari et al., 2005; Jha et al., 2007).

1.3.8.4 Type III Secretion System (TIIISS)
Type III secretion system (TIIISS) represents a complex, supramolecular structure which traverses inner membrane, periplasmic space, outer membrane, extracellular space and a host cellular membrane. TIIISS is thought to be structurally and evolutionarily related to the flagella system (Gerlach et al., 2007). TIIISSs have been studied in several Gram-negative bacteria including Salmonella, Yersinia, Shigella, Escherichia, Pseudomonas and Xanthomonas. Effector proteins are directly injected into the host cell cytoplasm by this secretion system. TIIISSs are also termed as 'injectisomes' or 'molecular needles' because of their shape and ability to translocate protein in a cell contact-dependent manner (Cornelis, 2006).

The OM ring complex belongs to the secretin family. Needle length control is believed to be essential for the optimal function of TIIISS. In Yersinia, host cell contact is accomplished with the major adhesins YadA and Inv. For this reason, the TIIISS needle must span at least the distance determined by these surface molecules to interact with the target cell (Mota et al., 2005). In Shigella, it was shown that the length of lipopolysaccharide (LPS) moiety was evolutionary adapted for optimal TIIISS function (West et al., 2005) where the authors have suggested a correlation between LPS glycosylation and needle length.
A structure known as ‘needle extension’ was identified at the tip of the needle. This structure may be responsible for the formation of the translocation pore (Mueller et al., 2005). The translocation pore inserted into the host cell membrane consists of the ‘translocator’ proteins. The YopB and YopD proteins of Yersinia sp. are the best studied translocator proteins (Viboud et al., 2005).

1.3.8.5 Type IV secretion system
The Type IV secretion system (TIVSS) is involved in the translocation of proteins or protein complexes as well as single-stranded DNA. TIVSS is thought to be evolutionarily related to bacterial conjugation systems (Cascales et al., 2003). TIVSSs are categorized into two subclasses: type IVA and type IVB (Christie et al., 2000). The T-DNA transfer system of A. tumefaciense is the prototype of TIVA while the dot/icm system of Legionella pneumophila represents TIVB system (Vogel et al., 1999).

1.3.8.6 Type V secretion System
Perhaps the Type V Secretion system (TVSS) represents the simplest protein secretion system described so far. Members of this group participate in a wide variety of functions including adhesion, proteolysis, serum resistance, cell to cell spread and invasion (Henderson et al., 2004). TVSS has been categorized into three sub-groups: the autotransporter system (AT-1 or Type Va), the two-partner secretion system (TPS or Type Vb) and the oligomeric coiled-coil adhesin system (Oca or AT-2 or Type Vc). Brief descriptions about these sub-groups are as follows:

A. The Autotransporter system (AT-1 or Type Va): AT-1 proteins are translated as a large multidomain precursor consisting of an N-terminal Sec-dependent signal sequence; an internal passenger domain, or α-domain which harbors the specific effector function; and a C-terminal translocation unit or β-domain. Following transport across the IM and cleavage of the signal sequence, the C-terminal domain inserts into the OM and forms a β-barrel secondary structure through which, in turn, the passenger domain is exported.
to the cell surface. Thereafter, the passenger domain may either remain attached to the bacterial cell or be released to the external milieu. Pohlner and associates (1995) were the first to describe and propose a model for Type V secretion, based on their studies on the gonococcal immunoglobulin A1 (IgA1) protease and its extracellular product (Pohlner et al., 1987).

B. The two-partner secretion system (TPS or Type Vb): In this family, the passenger domain and the transporter domain are translated as two independent polypeptides which are referred to as members of the TpsA and TpsB families, respectively (Jacob-dubuisson et al., 2004).

C. The oligomeric coiled-coil adhesin system (Oca or AT-2 or Type Vc): The salient features of this system are formation of oligomeric (often trimeric) complexes at the bacterial surface and formation of a β-barrel pore at the C-terminal ends. The oligomeric complex is required for formation of the β-barrel pore. Several afimbrial adhesins belong to this group. YadA of Yersinia sp. is one of the best characterized members of this system (Roggenkamp et al., 2003).

1.3.8.7 Chaperon usher (CU) pathway
Chaperon usher (CU) pathway is involved in the assembly of certain adhesion related surface structures. Examples of this system are Type 1 pili and P pili of uropathogenic E. coli. The subunits are translated as pre-pilins and translocated into the periplasm by Sec-dependent machinery. The major components of this system are: an outer membrane located usher, periplasmic chaperon, filament sub-unit and a tip adhesin (Nuccio et al., 2007). In type I pilus, FimC acts as a periplasmic chaperone for other pilus subunits (Vetsch et al., 2004). The integral outer membrane protein, FimD acts as the assembly platform or usher which facilitates the translocation of folded subunits through a large pore (Saulino et al., 2000). Primarily based on the features of fimbrial usher protein (FUP), a classification system has recently been proposed by Nuccio and Baumler (Nuccio et al, 2007) where the authors have proposed three major divisions of CU pathway: Alternate,
Classical and Archaic chaperon/usher systems and six clades, namely, \( \alpha \) (alpha), \( \beta \) (beta), \( \gamma \) (gamma), \( \pi \) (pai), \( \kappa \) (kappa) and \( \sigma \) (sigma). Xoo chaperon/usher pathway has been categorised in the archaic division which has a single clade, sigma (\( \sigma \)).

1.3.8.8 Type VI secretion system

Type VI protein secretion system was discovered and proposed recently in *Vibrio cholerae* by Pukatzki and coworkers (Pukatzki et al., 2006). The authors have shown that 'vas' (virulence associated secretion) gene products in *V. cholerae* encode a novel protein secretion system which has been termed as the prototype of Type VI secretion system. The secreted proteins are devoid of N-terminal hydrophobic leader sequence. Mutations in this cluster resulted in reduced virulence of *V. cholerae* towards the model host, *Dictyostelium discoideum*. Mutation in Type VI secretion system in *Burkholderia mallei* has been found to reduce virulence on hamster (Schell et al., 2007).

1.3.9 Bacterial adhesins: classification, structure and functions

Bacterial adhesion to biotic and abiotic surfaces enhances bacterial survival and is a major virulence function of pathogenic bacteria. Adhesion is a fundamental step in bacterial pathogenesis. In the early stages of infection, bacteria colonize hosts by adhering to host cell surface or the extracellular matrix. Bacteria also exhibit adherence capability to inorganic objects that can penetrate the host, such as medical devices, and thus can be a potential factor to cause infection. The adhesion of multiple bacterial cells to each other, also called autoaggregation, also plays an important role in the development of bacterial communities, such as microcolony or biofilm, which can promote pathogenicity.

Adhesion process generally takes place by a specific receptor-ligand interaction where a ligand on the bacterial surface docks on the host surface receptor. These bacterial surface structures are specialized bacterial proteins
which are called bacterial adhesins. Depending upon the assembly mechanism and structure, all the adhesin molecules are sub-divided into two major groups: fimbrial adhesins which are component of a multi-protein complex and non-fimbrial/adfimbrial adhesins which act as individual protein molecules directly associated with the bacterial membrane (Soto et al., 1999).

A single bacterial pathogen can have different kinds of adhesins (Jacques et al., 1998; Ofek et al., 2003; Dunne Jr. WM, 2002). The reasons may be the need to make adhesion a failsafe mechanism by engaging different host receptors simultaneously or the necessity of binding to different receptors in the host at different stages of infection. Most of the present knowledge about the biosynthesis, structure and functions of adhesins stem from studies on human and other animal pathogenic bacteria.

**A. Fimbrial adhesins**

Fimbriae, also called pili, are filamentous and heteromeric protein structures which are present on the bacterial surface. Usually only one of the components of the fimbriae acts as an adhesin. Fimbrial adhesins usually act in a receptor specific manner, however, in some cases, exhibit nonspecific adhesion by increasing the surface hydrophobicity of the bacteria (Lindahl et al., 1981). The major fimbrial adhesins are described as follows:

**Type I and P pili**

Type I pili and Pyelonephritis-associated (P) pili are assembled by the CU pathway. Both are found in uropathogenic *E. coli* (UPEC) but exhibit difference in binding specificities. The *fim* gene cluster encodes type I pili, in which FimC is the periplasmic chaperon, FimD is the outer membrane usher, and FimH is the pilus adhesin. FimH variants present on non-pathogenic *E. coli* show high affinity to tri-mannose containing glycoprotein receptors while UPEC express predominantly FimH variants with high affinity to mono-mannose residues. FimH mediated receptor binding was shown to cause bacterial internalization within bladder cells resulting in persistence and chronic urinary tract infections (Martinez et al., 2000). It has been shown that
the gross binding specificity is determined by both adhesive tip fibrillum and the pilus shaft (Duncan et al., 2005). *Salmonella enterica* serovar typhimurium type I pili are encoded by the *fim* gene cluster and can adhere to HeLa cells (Baumler et al., 1996).

P pili are encoded by *pap* gene cluster in pyelonephritic *E. coli* and represent the prototype for the chaperon/usher pathway. It consists of six structural proteins and is composed of two distinct sub-assemblies (Li et al., 2004). The PapG subunit is located at the distal end of the tip fibrillum and binds to Galα(1-4)Gal moieties which are present in glycolipids of kidney. The PapG protein is joined to the tip fibrillum (PapE) by an adaptor protein called PapF. The tip fibrillum (PapE) is joined by the PapK protein to the PapA rod. The rod is terminated by PapH, which may also act to anchor the pilus in the membrane. PapD is the periplasmic chaperon and PapC is the OM usher for P pili assembly. Host and tissue specificity varies due to the presence of different PapG adhesins recognizing different but related Galα-(1-4)-Gal sugars (Feria et al., 2001).

**Afa/Dr adhesin**

Afa/Dr adhesins (diffuse adherence fibrillar adhesin/Dr blood group antigen) include both fimbrial and afimbrial adhesins which assembled via the CU pathway. In DAEC (diffusely adhering *E. coli*) members, Afa/Dr family operon consists of at least five *afa* genes (*afaA* to *afaE*) which encode transcriptional regulator, a periplasmic chaperone, an OM-anchoring protein, an invasin and the adhesin, *afaE* (Servin, 2005).

**CS pili**

The coli surface antigen 1 (CS1) pili represents another distinct group of fimbrial adhesins. CS1 pili of enterotoxigenic *E. coli* show high functional similarities to the chaperone/usher pathway but there is little protein sequence similarity. So the CS pili assembly system is also termed as the ‘alternate CU pathway’ (Soto et al., 1999). An operon of four genes, *cooABCD*, is involved in the biogenesis of CS pili and all four proteins are transported into the
periplasm by GSP. The periplasmic chaperone CooB acts on CooA, C and D. The minor pilin CooD is believed to initiate pilus assembly and is located on the pilus tip. The OMP CooC is the probable usher through which CooD and the major pilin CooA are secreted (Starks et al., 2006). Mutational analyses have shown that adhesion as well as hemagglutination critically depends on CooD (Sakellaris et al., 1999).

Curli

Curli or thin aggregative fibers (agf) is another category of fimbrial adhesins. These adhesins have been reported in Salmonella sp. and E. coli strains. Expression of two operons, csgDEFG and csgBA regulate the expression of curli in these two organisms (Hammar et al., 1995; Romling et al., 1998). Both, biofilm formation and adhesion are regulated by curli (Brombacher et al., 2003). Bacterial adhesion to the human matrix proteins fibronectin, laminin, plasminogen, contact phase proteins and major histocompatibility complex class I molecules has been found to be mediated by curli (Ben Nasr et al., 1996; Olsen et al., 1998; Robinson et al., 2006). Assembly of curli at the bacterial cell wall has been reported to take place by extracellular nucleation precipitation (Hammar et al., 1996).

Type IV pilus

Type IV pilus are multiprotein filamentous cell appendages assembled primarily from a single protein sub-unit called pilin. The assembled pilus structure is extended through the OM and forms long and flexible surface appendages (Craig et al., 2004). Components of the type IV pilus assembly machinery are structurally related to TIISS, where homologous proteins are called ‘pseudopilins’. Type IV pilins share some common features: a short, positively charged leader peptide which is later cleaved off the prepilin, a conserved stretch of 25 hydrophobic residues, an unusual N-methylation at N-terminus and a disulfide-bond at the C-terminus (D region). Based on the sequence features, type IV pilins are grouped into two sub-classes, type IVa and type IVb (Craig et al., 2004). The two groups differ in some major characteristics, such as: the average length of the mature pilin protein is 150
aa in type IVa pilin but in type IVb, it is about 190 aa; N-methylated terminal residue is phenylalanine in type IVa pilin but in type IVb, it may be methionine, leucine or valine; average length of the D-region (the region that is bound by the two conserved C-terminal cysteines which form a disulphide bridge) is 22 and 55 aa for type IVa and type IVb pilin, respectively. Thousands of copies of pilin monomers assemble to form extremely thin (50-80 Å) filaments which are several microns in length. In spite of such narrow diameter, type IV pili are remarkably very strong – capable of withstanding stress forces greater than 100pN (Maier et al., 2002). Interactions between the N-terminal α-helices of pilins form the hydrophobic core and the C-terminus is exposed to the pilus surface. This hypervariable highly exposed surface is the source of strain specific antigenic variations and interactions with host surfaces (Craig et al., 2004). Additional proteins also may be located on the tip of the filament. In Neisseria gonorrhoeae, PilC is located at the tip of the filament and was shown to be acting as an adhesin (Rudel et al., 1995; Kirchner et al., 2005).

The involvement of type IV pilus in diverse cellular functions such as, bacterial adhesion and colonization to host surface, virulence functions, modulation of target cell specificity, bacteriophage adsorption, twitching motility, transformation competence and social gliding etc have been documented primarily on different animal/human-bacteria interactions and also for bacteria-bacteria interactions. In the diazotroph, Azoarcus sp. BH72, PilA has been shown to be essential for pilus formation and affects colonization of plant roots as well as fungal surfaces (Juliane et al., 1998) thus providing a good example of a more general role of type IV pilus in plant-bacteria and fungus-bacteria interactions.

PAK pili represent one of the best characterized examples of type IVa pili. PAK pili, the major adhesin of Pseudomonas aeruginosa strain K, were shown to mediate adhesion to mucosal epithelial cells by binding to the glycolipids asalio-GM1 and asalio-GM2 (Krivan et al., 1988). Neisseria sp. contains GC pilin and uses PilC, a 110-kDa protein localized at the pilus tip, as adhesin to
bind to the transmembrane glycoprotein CD46, which is present on all human cells except erythrocytes and is involved in complement activation (Kirchner et al., 2005).

Interestingly, type IVb pili have been found primarily in bacteria which are able to colonize the human intestine. In V. cholerae, the toxin-coregulated pilus was shown to be involved in adhesion and autoaggregation (Kirk et al., 2005). Type IVb PilS of Salmonella enterica serovar typhi binds to human intestinal epithelial cells (Zhang et al., 2000) where cystic fibrosis transmembrane conductance regulator (CFTR) acts as the receptor (Pier et al., 1998).

Type IV pili are also termed as retractile type IV pili as it can retract itself while the pilus tip is still attached to the substratum. Pilus retraction is required for a specialized movement across semisolid matrices (e.g. mucosal epithelia) called 'twitching motility' (Burrows, 2005) as seen in Pseudomonas, Neisseria while called gliding motility in Myxococcus xanthus. Twitching motility was first coined by Lautrop (1961) to describe flagella-independent surface motility in Acinobacter calcoacetius (Lautrop, 1961) and investigated in detail in Psedomonas aeruginosa (Bradely, 1980). Such motility appears to be a means of rapid colonization by bacterial species of surfaces of high nutrient availability. It occurs on wet surfaces and is important for host colonization, formation of biofilm and fruiting bodies (Mattick, 2002). Type IV pilus mediated twitching motility is thought to be involved in the migration of Xylella fastidiosa through xylem vessels, in a direction that is opposite to the movement of the transpirational stream (Meng et al., 2005).

B. Afimbrial adhesins
Afimbrial adhesins constitute a large family of mono- or oligomeric surface-associated proteins. The members of this family can be divided in the following groups: autotransported adhesins, integral outer membrane adhesins, secreted adhesins and adhesins associated with biofilm formation (Gerlach et al., 2007).
**Autotransported adhesins:** Autotransporters are a large and diverse superfamily of proteins produced by pathogenic Gram-negative bacteria and are secreted by Type V secretion system. Since the first autotransporter was identified in *Neisseria gonorrhoeae* as IgA protease (Pohln et al., 1987), more than 800 putative autotransporters have been identified in the bacterial genome sequences to date (Dautin et al., 2007).

Autotransporters have a modular organization with an N-terminal signal peptide, an internal passenger domain harbouring the function of the protein and a C-terminus translocation unit or β-domain. Biogenesis of the autotransporters occurs through multiple steps. The first step of secretion involves the crossing of the inner membrane in a Sec-dependent manner. The second step involves the insertion of the translocation unit in the outer membrane and finally, the passenger domain is translocated through the translocation unit across the outer membrane. Once on the bacterial surface, some autotransporters may undergo additional cleavage event(s) that separate the passenger unit from the translocation unit.

Further study has led to the discovery of two variations in the nature of autotransporters. In one case, the translocation unit consists of approximately 300aa and is predicted to form a 12-stranded β-barrel, proceeded by an α-helix and the passenger domain. These autotransporters are called ‘Conventional autotransporters’ which are mostly monomeric. In another case, the translocation unit is much shorter and is predicted to encompass only 4 β-strands, immediately preceded by the passenger domain. These autotransporters have been shown to form homotrimers. Thus, the functional translocator is a 12-stranded β-barrel. The passenger domains also fold as trimers and, therefore, this distinct sub-family is termed as ‘Trimeric autotransporters’ (Cotter et al., 2005), the prototype being YadA of *Yersinia* sp. Another interesting difference among these two sub-groups is the presence of an autochaperon region at the C-terminal end of the passenger domain in the conventional autotransporters (Oliver et al., 2003). Again, some
autotransporters (e.g., *E. coli* AIDA, TibA) may undergo glycosylation in the passenger domain (Bentz et al., 2001; Lindenthal et al., 1999).

The conventional autotransporters show a remarkable variety of effector functions which include adhesion, invasion, autoaggregation, proteolysis, cytotoxicity, serum resistance, cell to cell spread etc (Girard et al., 2006). Interestingly all the trimeric autotransporters functionally characterized have been found to be acting as adhesins (Cotter et al., 2005) and hence the name 'trimeric autotransporter adhesins' (TAAs). Structural studies show a general pattern in all the TAAs resembling a lollipop consisting of head domain which possibly harbours the adhesin function, the neck, the stalk and anchor domain (Girard et al., 2006). The head and stalk are composed of many repetitive domains which may be involved in recombination of domains resulting in the variations of specificity of adhesins (Linke et al., 2006).

There are diverse cellular receptors that have been identified for autotransporter adhesins. One example is *Helicobacter pylori* adhesins BabA and SabA that bind to human Lewis blood group antigens which are expressed on gastric epithelial cells (Ilver et al., 1998; Mahdavi et al., 2002). Receptors for many autotransporter adhesins have been found to correspond to extracellular matrix proteins such as collagen, fibronectin, or vitronectin (Tahir et al., 2000; Tertti et al., 1992; Tamm et al., 1993).

The regulation of expression of the autotransporters has been studied in some members. It may be of various kinds: temperature dependent expression (UspA1 and YadA), availability of iron (NadA regulated by Fur), minimal media dependent expression (XadA of Xoo) (Heiniger et al., 2005; Skurnik et al., 1992; Martin et al., 2005; Ray et al., 2002).

Many autotransporters have been found to be polarly localized. For example, IcsA autotransporter fused with GFP was found to be localized in one pole of the cell in a variety of enterobacteria and *V. cholerae* (Charles et al., 2001). A recent study demonstrated that a variety of autotransporters including AIDA-I,
BrkA, and the *Shigella flexneri* SepA protein also exhibit a unipolar localization both in native hosts and *E. coli* (Jain et al., 2006). NalP is unipolar in *E. coli* but is localized to multiple distinct foci in *Neisseria meningitidis*, which is a spherical organism and thus does not have any distinct poles (Jain et al., 2006).

**Integral outer membrane (OM) adhesins:** Several outer membrane proteins (OMPs) having β-barrel domain exhibit adhesion functions (Niemann et al., 2004). *E. coli* OmpA plays a vital role in causing meningitis in neonates. Ecgp which is present on the surface of human brain microvascular endothelial cells acts as the receptor of OmpA (Prasadarao, 2002). OpcA, an OMP of *Neisseria meningitidis* mediates bacterial adhesion and invasion of human epithelial and endothelial cells (Virji et al., 1992).

**Secreted adhesins:** Many proteins secreted by TISS have been found to be involved in calcium ion binding and harbour glycine rich repeats (GGXGXDXXX). These proteins have been predicted to act as adhesins (Delepelaire, 2004). Several secreted proteins have been reported to have adhesion functions, such as, LapA of *Pseudomonas fluorescens* (Hinsa et al., 2003), SiiE in *Salmonella enterica* (Gerlach et al., 2007), GbpA in *Vibrio cholerae* (Kirn et al., 2005). However, there is no detailed report about the significance of the action of secreted adhesins.

**Adhesins associated with biofilm formation:** Biofilm formation is often an important factor for microbial colonization and virulence. A strong interaction between adhesins and receptors is considered to be an important factor for biofilm formation. A notable example of this category is the BAP (Biofilm Associated Proteins) family of adhesins. These adhesins are present both in Gram-positive as well as in Gram-negative species and all members have a high molecular mass, a signal sequence for extracellular secretion and a repetitive structure. *Staphylococcus aureus* Bap (2276 amino acids long) is the prototype of this family and has an N-terminal secretion signal, a long repetitive central moiety and a C-terminal cell wall-attachment region.
comprising an LPXTG motif (Cucarella et al., 2001). *Salmonella Typhimurium*, BapA is another BAP family member and is important for biofilm formation. BapA expression is regulated by CsgD which is considered to be acting as the global regulator of biofilm formation in *Salmonella* strains (Latasa et al., 2005).

1.4 Theory of bacterial adhesion

Microbial adhesion involves transformation of a free cell to a firm attached state over a surface (Hermansson M., 1999). The process of bacterial adhesion to an available surface is governed by several variables, including the species of bacteria, surface composition, environmental factors, and essential gene products. Adhesion between bacteria and abiotic surfaces is generally mediated by nonspecific (e.g., hydrophobic) interactions, whereas adhesion to living surface involves specific molecules such as lectin, ligand, or adhesin. Bacterial adhesion to a surface can be described as a two-phase process (Marshall et al., 1971) which was first realized by ZoBell who suggested that once bacteria are attracted to the surface, firm attachment requires incubation for several hours (Zobell, 1943). Phase one, also known as docking phase, involves primary and reversible adhesion process which involves physiochemical interactions. Phase two, also known as locking phase, involves secondary and irreversible adhesion processes which involve interactions between bacterial and host molecules. The later phase has been suggested to be time and energy dependent.

Several models have been put forward to describe bacterial adhesion that can be grouped in the following ways (Hermansson, 1999): the classical DLVO theory (Derjaguin–Landau–Verwey–Overbeek), cell surface hydrophobicity, thermodynamic approach, extended DLVO theory, conformational changes of protein due to adhesion, co-adhesion and biological factors. While none of the models can explain bacterial adhesion process completely, a synthetic approach to formulate a ‘unified theory of microbial adhesion’ may be a better option.
1.5 Laboratory methods for the study of bacterial adhesion to plants

Adhesion of bacteria to plants is thought to be a dynamic and complex interplay between bacteria and the plant surface. Therefore, the adhesion experiments should be carefully designed so that it can reflect the natural conditions to maximum effect. There are several factors which can influence the adhesion process significantly (Matthysse, 1995):

1. Plant material: It may be leaves, roots or root cap cells; intact tissues or wounded tissues; artificial systems, such as suspension culture or callus culture.

2. Plant physiology: It can modulate nutrient availability to bacteria as well as the composition of the surface. So photoperiod, flowering or stressed plants, mineral nutrition, plant growth condition, humidity are important variables.

3. Bacterial culture: Bacteria grown in rich complex medium may likely experience considerable nutritional stress when exposed to plant surface. Again, bacteria in exponential or stationary phase can show different adhesion properties.

Bacterial adhesion is often measured by counting the numbers of attached cells after a defined washing step that removes loosely adhered bacteria. Adhesion process can also be represented as a function of time or as a steady state after some defined time (Hermansson, 1999).

Several methods have been reported for the study of bacterial adhesion to plant surface. Based on the experimental objectives, all the methods can be grouped into three main classes (An et al., 1997):
1. Microscopy based methods:

A. Light microscopy: Light microscopy is an elementary approach for both bacterial counting and morphological observation. It offers a good tool for in vitro analysis based on translucent surface like glass slide. Bacteria may be observed without or with application of various stains/dyes. Special staining procedures may be applied for visualization of surface structures like capsules or flagella.

B. Fluorescence microscopy: For smooth and opaque surface as well as the natural substratum, use of fluorescent stains and marker molecules like green fluorescent protein (GFP) are widely used for both in vitro and in vivo experiments. With the advent of advanced image analysis systems, epifluorescence and confocal microscopy are being used extensively for determining the number of cells adhered, area coverage, and biovolume of attached cells in real time. This procedure is very quick, sensitive and can reduce the possibility of operator bias.

C. Scanning electron microscopy (SEM): SEM provides another basic method for the study of bacterial morphology following adherence on the surface and also the morphological features of the natural substrata. It also provides valuable information about the dual relationship.

D. Atomic force microscopy (AFM): Because of its piconewton force sensitivity and nanometer positional accuracy, the atomic force microscope (AFM) has emerged as a powerful tool for exploring the adhesion dynamics. It can not only image bacterial adhesion to different substrata in several in vitro assays but also provides valuable information about the receptor-ligand interaction by measuring the absolute force involved in the process (Stroh et al., 2004). Functional probe tip of the AFM system can be used to find out the possible host receptor of the bacterial adhesins. Absolute adhesion forces involving multiple intermolecular bonds between the heparin-binding hemagglutinin adhesin (HBHA) produced by Mycobacterium tuberculosis and heparin have
been calculated by AFM and the distribution of HBHA molecules have been shown to be concentrated into nanodomains on the surface of living mycobacteria (Dupres et al., 2005).

2. Viable bacterial counting method:

A. CFU (Colony forming unit) plate counting: This is perhaps the most basic method for bacterial counting. There are two basic ways for CFU counting method (Herbert, 1990) - the pour plate and surface spread method. Modifications are available and by crushing the inoculated plant materials, dilution platting is done. Following an incubation period, the cell number is counted.

B. Radiolabelling: The method involves initial labelling of bacterial cells with radiotracers such as $^{14}$C leucine, $^{14}$C glucose, $^3$H thymidine or $^3$H palmitic acid. Then the adhesion process is followed by scintillation counting. This method is very useful for irregular surface, highly sensitive, accurate and rapid. But the potential risk to the human health and high cost are the main disadvantages.

C. CTC staining: The method provides an estimate of metabolically active cells. The fluorescent redox dye, 5-cyano-2',3-ditolyl tetrazolium chloride (CTC) is used as an indicator for electron transport activity. When oxidized, CTC is colourless and does not fluoresce, but when reduced by electron transport activity, the dye forms fluorescent CTC-formazan which is imaged by fluorescence microscopy.

3. Other methods:

A. Spectrophotometry: It's based on the quantitative relation between optical density and colony counts being derived from standard curves prepared for each bacterial species. In this method, either the substratum with adhered bacteria is examined directly after staining (e.g., crystal violet, Congo red etc)
or, alternatively, bacteria are washed off the substrata, stained and then the solution is examined in a spectrophotometer.

B. Coulter counter: This has a limited usage for counting and measuring the size of bacteria. It calculates the resistance of a conducting solution as a particle passes through an aperture.

C. Biochemical markers: Several metabolites have been reported to help in understanding the process of bacterial adhesion. Use of bioluminescence ATP to assess bacterial adhesion with hydrophobic polystyrene has been reported (Harber et al., 1983). Quantitation of bacteria was done by measuring the amount of total ATP present in bacteria adhering to pieces of a synthetic polymer (Ludwicka et al., 1985). Number of adhered bacterial cells has also been evaluated by measuring their DNA content with a fluorometric technique using Hoechst staining (Paul et al., 1993). Cellular urease has been used as a marker to measure the adhesion of *Staphylococcus epidermidis* to plastic (Dunne et al., 1991). Measurement of lipopolysaccharide (LPS) content as a marker for attached bacteria was also documented (Watson et al., 1977).

D. Immunofluorescence microscopy: It involves generation of specific antibodies and use of fluorescent conjugates to detect surface attached bacteria. Observation is done under a fluorescence microscope (Campbell et al., 1983; Dahle et al., 1982). This method can identify specific bacteria even in a cluster of different bacterial species.

E. Microfluidic/parallel plate flow chambers: These are useful tools for measuring adhesion force and to elucidate the adhesion property of adhesin mutants. In *X. fastidiosa*, the role of type I fimbriae and type IV pilus has been demonstrated for glass substratum adhesion using this technique (Fuente et al., 2007). The role of FimH of *E. coli* in glass adhesion was shown to be shear stress dependent using such parallel flow chamber (Thomas et al., 2004).
F. Laser tweezers: Certain biophysical techniques like use of laser tweezers have been applied in microbiology to study dynamics, force generation and even enzymatic cycles of molecular motor proteins to a great resolution (Maier, 2005). Laser tweezers can trap objects as small as 5nm and exerts force exceeding 100pN. The nanomechanics of adhesion and twitching motility in *N. gonorrhoeae* mediated by type IV pilus have been documented by using this technique (Merz et al., 2000).

1.6 Bacterial adhesion to plant surfaces

Successful development of a plant disease requires multiple successive steps which start with the adhesion of the pathogen to the plant surface, penetration/entry of the host tissue, and the multiplication of the pathogen inside the host plant after entry. It is evident that adhesion of the pathogenic bacteria to susceptible plant is an essential initial step in the infection process. There are three major aspects of this adhesion process (Romantschuk, 1992):

A. Forces involved in bacteria-plant interactions

Initial binding of bacterial cells to a plant surface involves a reversible, non-specific phase which is followed by irreversible, specific phase. Because of a net-negative surface charge of both bacteria and plant, electrostatic forces may inhibit a close contact between the host and the pathogen. Therefore, the bacterial cell has to overcome this repulsive energy barrier for adhesion to plant surfaces. This may be achieved by local positively charged groups on either of the surfaces and also by hydrogen bonds and van der Waals force of attraction. Additionally, hydrophobic molecules on the plant surface and hydrophobic regions of the bacterial surface proteins may promote close interactions.

B. Specificity for host and site in adhesion

Bacterial pathogen shows specificity for both host and the site of infection. The host probably provides pathogenic bacteria the best naturally occurring niche. Furthermore, in a host plant, pathogens may exhibit tissue specificity for the adhesion and entry. For example, *Pseudomonas syringe* pv.
phaseolicola adheres preferentially to the stomata of bean (Romantschuk et al., 1986). Interestingly, some bean- and corn-pathogenic strains of \textit{P. syringe} pv. syringe distribute evenly over the leaf surface of both susceptible and heterologous plants (Mills et al., 1991). Both, \textit{Xanthomonas campestris} pv. hyacinthi and isolated fimbriae were found to attach preferentially to the hyacinth stomata (van Doorn et al., 1994). The nitrogen fixing \textit{Klebsiella} sp. adheres specifically to the root hairs (Haahtela et al., 1986).

C. bacterial adhesins and virulence

Our present knowledge on adhesins of plant pathogenic bacteria and target sites on the host are very limited at the molecular level. Although extensive literature is available on the correlation between adhesion and virulence on the animal pathogenic bacteria, very little information is available about the adhesins of plant pathogenic bacteria. All the bacterial molecular structures involved in adhesion till date can be grouped in the following ways, primarily depending on their composition: proteins (adhesins), polysaccharides and small soluble molecules.

Proteins

Bacterial adhesins may be either fimbrial or afimbrial proteins as described earlier. While in the afimbrial adhesins, some portions of the single protein may specifically act as binding domains while in fimbrial adhesins, one or more subunits interact with the receptors on the target surface. Isolated fimbriae and fimbriated cells of \textit{Erwinia rhapontici} which infects rhubarb (\textit{Rheum rhaponticum}) and causes pink wheat grains, efficiently adhere to rhubarb leaf surface and wheat grain (Louhelainen et al., 1990). It was also found that adhesion to and infection rate of wheat grains by \textit{E. rhapontici} were reduced in the presence of \(\beta\)-galactosidase indicating the role of a \(\beta\)-galactoside moiety in binding (Korhonen et al., 1988). Type I fimbriae are widespread among the members of Enterobacteriaceae and are mannosides specific (Jann et al., 1990). Fimbriae of \textit{Klebsiella} sp. were suggested to cause adhesion of bacterial cells to the root hairs of \textit{Poa pratensis} (Haahtela et al., 1985). Several strains of \textit{P. syringe} express pili which function as
receptor for bacteriophage φ6 (Romantschuk, 1986). Pilus-negative, spontaneous and transposon induced, mutants of various pathovars of *P. syringe* exhibit greatly reduced adherence to both the leaf surface of susceptible host and non-susceptible plants (Nurmiaho et al., 1991; Romanstchuk et al., 1986). *P. syringe* pv. phaseolicola, the causal agent of halo blight of bean, enters inside the leaf through the stomata. It has been shown that piliated bacterial cells specifically attach to the stomata while non-piliated cells adhere in lower number throughout the leaf surface. As a direct correlation between adhesion and virulence, it was also demonstrated that both piliated and non-piliated cells cause the disease when the bacterial cells are directly introduced inside the leaves but when spray-inoculated, only piliated strains can cause the disease and the non-piliated strains are not able to cause halo blight symptoms (Romantschuk et al., 1985). Studies indicate that pili probably play a significant role in the attachment of *Bradyrhizobium japonicum* to soybean roots. Treatment of *B. japonicum* suspensions with antiserum against the isolated pili reduced attachment to soybean roots by about 90% and nodulation by about 80% (Vesper et al., 1986).

*Erwinia chrysanthemi* which causes soft rot disease on a wide variety of plants including tobacco harbors an adhesin protein called HecA (hemolysin-like protein in *E. chrysanthemi*) which belongs to *Bordetella pertussis* filamentous hemagglutinin family of adhesin. A transposon induced mutant of hecA shows defects in adhesion, aggregation and epidermal cell killing. All these three events occur sequentially at the early stage of infection much before the development of symptoms. Very interestingly at the later part of the infection process the mutant exhibited reduced virulence on tobacco seedlings after inoculation without wounding which indicates a direct correlation between adhesion and virulence (Rojas et al., 2002).

*Xylella fastidiosa* is a Gram-negative, xylem-limited bacterium and causes several economically important diseases including Pierces Diseases (PD) of grapevine and citrus variegated chlorosis (CVC). The PD *Xylella fastidiosa* genome contains multiple putative fimbrial and afimbrial adhesins. It has been
shown that the expression of a fimbriae gene, fimA, is upregulated in freshly isolated cells of X. fastidiosa from citrus as compared to that of cells which were axenically cultured for several passages (de Souza et al., 2004). However, mutations in fimA and fimF did not alter the pathogenicity although there was a reduction in cell-to-cell aggregation and cell size (Feil et al., 2003). It has been shown that HxfA and HxfB hemagglutinin proteins are involved in X. fastidiosa self-aggregation. Interestingly, mutants that are defective in these proteins are hypervirulent (Guilhabert et al., 2005). It has been proposed that migration of X. fastidiosa through the xylem vessels is hindered by the aggregates of X. fastidiosa cells that are formed due to action of HxfA and HxfB. The hypervirulent nature of the mutants placed these hemagglutinin genes in a novel anti-virulence gene category. Recently the role of X. fastidiosa adhesins in biofilm formation was also studied (Feil et al., 2007). It was shown that fimbrial mutants, FimA- and FimF- adhered to glass surface as solitary cells and in a slightly lesser frequency compared to wild type but don't show cell aggregates while afimbrial mutants XadA- and HxfB- show much lower frequency of adherence and are found mostly in cell aggregates of different sizes like the wild type. The significance of this observation may indicate a functional territory for afimbrial and fimbrial adhesins; while fimbrial adhesin participate in self-aggregation of the bacterial cells, the afimbrial adhesins primarily participate in substrate adhesion.

Both tomato and Arabidopsis are susceptible to crown gall tumor formation by Agrobacterium tumefaciens. Studies suggested that attachment genes in A. tumefaciens play a role in host tissue attachment and also in virulence. Mutations in the genes attR, attB and attD have been reported to reduce the bacterial colonization on the host tissues (Matthysse et al., 1996; Reuhs et al., 1997; Matthysse et al., 1998). Mutation in attR affects the production of a surface associated acidic polysaccharide while attB and attD gene products participate in the plant-bacteria signaling. Plant pathogenic A. tumefaciens and many other members of the family Rhizobiacea mediate direct attachment to the plant root hair cells through Ca$^{2+}$ dependent adhesin called rhicadhesin (Smit et al., 1989). Interestingly, Xanthomonas campestris pv.
vesiculatoria, mutation in fimA does not affect bacterial adhesion to tomato leaves and virulence (Ojanen-Reuhs et al., 1997).

Fibrilar structure
Fibrilar structures, presumably composed of bacterial cellulose have been reported to be involved in the formation of large bacterial clusters of both Agrobacterium and Rhizobium. Agrobacterium mutants lacking cellulose exhibits reduced virulence (Minnemeyer et al., 1991) and absence of large bacterial clusters (Mathysse, 1983).

Small soluble molecules
It has been shown that mutations in chromosomal genes chvA, chvB and exoC/pscA of Agrobacterium affect the virulence property and the mutants are unable to attach to plant cells (Cangelosi et al., 1987; Douglas et al., 1985; Thomashow et al., 1987; Swart et al, 1994). chvB and exoC/pscA are required for synthesis (Cangelosi et al., 1987) and chvA is required for export of the low molecular weight cyclic β-1,2-glucan molecule (Cangelosi et al., 1989).

1.7 Entry of bacteria inside the host plants

Aerial plant organs, particularly leaf tissues, provide an excellent niche for microbial colonization. Upon introduction to leaf surface, potential bacterial pathogens survive an initial epiphytic phase but entry of bacterial cells inside the leaf tissue is an absolute requirement to initiate the pathogenesis. To gain access to the internal leaf tissue, microbial pathogens need to cross the cuticle and epidermal layer. Bacterial pathogen cannot directly penetrate the leaf surface as contrast to the fungal pathogens and instead plant pathogenic bacteria exploit natural openings and wounds to gain entry inside the host tissues. All these ports of entry can be classified into three major categories: natural openings, injuries/wounds and intact surface (Huang, 1986). A brief discussion about the different modes of bacterial entry has been presented below:
A. Natural openings

Majority of plant pathogenic bacteria enter inside the plant tissue through natural openings situated on the plant surface. These structures include stomata, hydathodes, nectarthodes, lenticels etc. Recent studies have shown that the process of bacterial entry inside the leaf tissue through natural openings like stomata is complex and dynamic in nature.

**Stomata:** Among all the natural openings, stomata predominate in number and primarily occur on surfaces of leaves and young stems. A stoma usually consists of a pair of specialized epidermal cells called guard cells. The epidermal cells adjacent to the guard cells are called subsidiary cells. The internal space defined by the guard cells, the subsidiary cells and the neighbouring cells form the sub-stomatal cavity. The opening and closing of the cavity is highly regulated by the guard cells and it depends on several factors like photoperiod, humidity, CO₂ concentration, stress etc. The major function of the stomata is to maintain the gaseous exchange during photosynthesis and water balance through transpiration. Various studies suggest that stomatal number, size, structure and morphology may influence disease resistance (McLean, 1921; Vantuyl et al., 1982).

It has been found out that several species of plant pathogenic bacteria, such as, *Pseudomonas* can enter the plant tissues through stomata. However, the stomata do not act as a passive port of entry as thought earlier. Recently it has been shown that stomata can also act in host innate immune response (Mellotto et al., 2006). The authors in their study on *Arabidopsis-Pseudomonas syringae* system, have shown that perception of the pathogen by the host leads to the stomatal closure. However, pathogenic strains are able to reopen the closed stomata. It has been shown that the bacteria can secrete a phytotoxin due to which stomata again open allowing the entry of the pathogen.

**Hydathodes:** Hydathodes are structurally very similar to stomata but the associated guard cells are not functional to regulate the opening/closing of the apertures which always remain open. These structures are usually present on
the margins of leaves or at the very leaf tips on the upper (dorsal) surface of leaves. Hydathodes are present in several plant families including Gramineae, Crassulaceae, Saxifragaceae and Brassicaceae (Huang, 1986). The major function of hydathodes is secretion of guttation droplets which are formed from the xylem fluid and secreted in the morning hours (Curtis, 1943). The droplets are continuous with the xylem fluid and later on as the day progresses and temperature increases, are drawn back inside the tissues. It is assumed that bacteria which are suspended in the guttation fluid may gain entry inside the leaf in this way. Interestingly, the guttation fluid has been reported to contain trace amount of substances which may act as chemoattractant as well as nutrient source for pathogenic bacteria (Chet et al., 1973; Feng et al., 1975).

*Xanthomonas campestris* pv. *campestris* (Xcc), the causal organism of black-rot disease of many crucifers, was reported as the first plant pathogenic bacteria to enter through hydathodes (Curtis, 1943). Xoo which causes bacterial leaf blight of rice also exhibits hydathodal mode of entry (Tabei, 1967).

It has been observed that certain structural features of the hydathodal guard cells may contribute to the resistance against Xoo invasion. *Leersia japonica*, a resistant wild rice, has hydathodal apertures of about 0.9µm which is too small for Xoo to enter inside the hydathodal cavity. In susceptible *Oryza sativa*, these are much larger (about 2.9µm) which is enough for Xoo to enter inside the leaf (Horino, O., 1983; Horino, O., 1984).

One recent report provides evidence that plants have also evolved some active mechanisms to protect themselves from the pathogenic invasion through the hydathodes and guttation fluids. It has been shown that the guttation fluid collected from uninoculated barley seedlings contains several proteins and out of about 200 proteins analyzed, many are pathogenesis-related proteins (PR proteins) (Grunwald et al., 2003) whose function may be to protect plants against pathogens.
**Nectarthodes:** Nectarthodes are stomata like structures through which droplets of nectar are excreted (Rosen, H.R., 1936). It consists of a pair of guard cells without the regulatory function of aperture opening. Cuticle covers entire nectarial surface and the nectarthodes are the only region where cuticle is disrupted. A specialized tissue which is below the nectarial region produces the nectar. Nectarthodes are found in an open space between the basal part of the styles and stamens (Huang, 1986). These structures are commonly present in the blossoms of apple, pear and many ornamental plants of Rosaceae. *Erwinia amylovora*, which causes fire blight of apple, pear and many ornamental plants of Rosaceae like *Cydonia, Mespilus* etc, enter through nectarthodes, multiply rapidly and eventually lead to the death of the entire blossom (Buban et al., 2003). Certain strains of *P. syringe* also infect pear blossom and cause blossom blight (Panagopoulos et al., 1963). *E. chrysanthemi* which causes fruit collapse of pineapple is also thought to cause disease in the similar mode (Lim et al., 1978).

**Lenticels:** Lenticels are composed of loosely packed cells which are present primarily in the periderm of stems and roots, often below a stoma. It appears on the surface as a lenticular (lens-shaped) spot which acts as a pore. Lenticels are usually of stomatal origin and function in gas exchange. During the transformation of stomata into lenticels, cells in the first two sub epidermal layers around the sub-stomatal cavity divide in both directions. It results in the formation of a mass of loosely arranged parenchyma cells with large intercellular spaces. Because of the continuity of the intercellular spaces of the lenticels and interior tissues, many pathogenic bacteria can enter through lenticels, such as, *Streptomyces scabies* (common scab of apple), *Erwinia carotovora* (soft rot of potato) (Huang JS, 1986; Fox et al, 1971).

**B. Entry through injuries and wounds**
Pathogenic bacteria may enter host plant through various sites on plant surface which have been exposed to wounds of various degree. Few common ways are described below.
**Broken Trichomes:** Trichomes represent one specialized group of epidermal projections. There are several variations in the forms, structures, and functions of trichomes. These structures are very fragile and may be either unicellular or multicellular, sometimes with secretory functions. Several bacterial species such as, *P. syringe* pv. *tomato*, *P. cichorii*, *Corynebacterium michiganensis* etc. have been found to grow on these structures and also to utilize these regions as a port of entry into the host plant (Schneider et al., 1977; Shirata et al., 1982; Layne RE, 1967). Bacterial multiplication takes place inside broken trichome bases which is followed by development of necrotic symptoms. Trichomes on ovaries are also gradually lost resulting the development of openings in the young fruit epidermis which may serve as infection sites (Getz et al., 1983).

**Emergence of lateral roots:** The source of origin of the lateral roots is usually the pericycle. It grows through the cortical region of the parent root. Crevices are formed when the lateral roots emerge outside by breaking the epidermal layer. Several reports suggest that such openings can be exploited by the pathogenic bacteria for entry inside the host plants. For instance, *Ralstonia solanacearum* (causes bacterial wilt disease in plants like tomato, eggplant, etc.) is capable of entering into tomato seedlings through this route (Schmit J., 1978).

**Wounds:** Several investigations about the role of natural and artificial wounds in bacterial disease development suggest that wounding on the plant surface may result in increased infection efficiency and virulence. *Agrobacterium tumefaciense* requires a wound to establish any infection. A positive correlation between the outbreak of bacterial blight of soybean caused by *P. syringe* pv. *glycinea* and rain with storms has been predicted (Daft et al., 1972). It was observed that storms and rain with strong wind may increase the disease severity. However, in some cases, wounding may reduce the infection process. Entry of *E. carotovora* subsp. atroseptica, the causal organism of blackleg of potato, through fresh wounds is generally lesser as compared to entry through potato lenticels. The possible reason may be
suberization and periderm formation in wound tissues that may create a barrier against the entry of the pathogen (Fox et al., 1971). But artificial wounding of host organs/tissues is also a common practice to understand the virulence property of the bacterial pathogens.

C. Entry through intact surfaces
It's a general observation that plant pathogenic bacteria do not possess penetration structures, such as penetration pegs commonly developed in the pathogenic fungi. But there are some reports which claim evidences for direct penetration of pathogenic bacteria into unwounded or intact plant tissue. One such report states that *E. chrysanthemi* is capable of infecting intact roots of *Dieffenbachia maculata* which are characterized by absence of lateral roots (Nieves-Brun, 1985), although intactness of the root was challenged by many workers. Some evidences of rhizobial penetration of root hairs of leguminous plants also suggest possibility of a direct penetration mode. In a study on *Trifolium repense-Rhizobium trifolii* interactions, it has been claimed that *R. trifolii* can gain entry into the root hair cells of *T. repense* by direct penetration which involves degradation of cell wall polysaccharides (Callaham, 1979 as mentioned by Huang, 1986).

1.8 Adhesion and entry of *Xanthomonas oryzae* pv. oryzae (Xoo) to rice leaves
It has been already mentioned that entry of the *Xanthomonas oryzae* pv. oryzae (Xoo), the causal agent of bacterial leaf blight of rice (BLB), inside the rice leaves, takes place through natural openings called hydathodes. Hydathodes are made up of water pores, the number of which varies from 10-20/hydathode in Taichung Native-1 (TN-1), a Xoo susceptible rice variety (Mew et al., 1984). Each water pore contains a pair of guard cells which are devoid of wax and papillae. The size of a hydathodal water pore is larger (2-4 times) than that of a stoma. Xoo cells on the leaf surface may become suspended in guttation fluid and are thought to enter the leaves as the fluid is withdrawn into the leaf (Curtis, 1943). The bacterial cells multiply in the underlying epithem, then enter and spread in the plant through xylem vessels.
Xoo, however, may gain entry also by wounds on the leaves. Inside the xylem vessel, Xoo cells may interact with and draw nutrients from the xylem parenchyma (Hilaire et al., 2001). In the leaves, Xoo cells migrate both vertically and laterally through primary and lateral veins, respectively. As the infection proceeds, Xoo cells may also ooze out of hydathodal pores which may act as a secondary source of inoculum. The initial symptoms of BLB as greenish streaks gradually merge to form yellowish lesions, often associated with wavy edges. The infection later on spreads through the leaf resulting in death of the entire leaf.

1.9 Summary

Bacterial adhesion to host surface is a crucial initial step for a successful infection process. Plant pathogenic bacteria have evolved several novel strategies to perform the function. It's clear that a single bacterial species may employ more than one strategy for host adhesion and it's more important when a pathogen has to encounter various tissue/cells of the host at different stages of infection. The plant pathogenic bacteria exhibit variations in the structures of adhesin-like functions. It may be a single protein like a conventional autotransporter to a complex of about several proteins such as Type IV pilus. With considerable amount of information from whole genome sequencing projects becoming available, the function of adhesion seems to be conserved in all the pathogenic bacteria. One remarkable feature is a very high similarity of sequence of adhesins between plant and animal pathogenic bacteria. It may imply a fundamental similarity in the bacterial pathogenesis of both plants and animals with regard to adhesion. Bacterial pathogens also exploit various ports of entry for gaining entry inside the host plants and the specificity of entry may be an underlying feature that is encoded in the genomes of bacterial pathogens.

In the last few decades, a lot of information has been gathered about the structure, biogenesis and functions of adhesins. However, most of the studies have been done on animal pathogenic bacteria. Comparatively much less efforts have been put forward to understand the significance of presence of
several putative adhesins in genomes of plant pathogenic bacteria. Finally, it's not mere adhesion and entry but the specificity with which the process takes place that is another feature of the host-pathogen interactions and a deeper understanding about the logic of adhesin functions may highlight an as yet little explored avenue of plant pathology.