1.1 Introduction

*Ralstonia* (previously known as *Pseudomonas* solanacearum) is a Gram-negative, plant pathogenic bacterium. It causes a lethal wilt disease in many plant species that include some common plants such as banana, cashew, eggplant, papaya, peanut, pepper, potato, tomato etc\(^1\). The bacterium is soil borne. In the presence of a suitable host plant, the bacterium invades its root tissue and reaches the xylem, where it colonizes. The bacterium spreads to the aerial parts of the infected plant through the xylem. Accumulation of a large number of bacteria in the xylem of the host results in wilting followed by death of the infected plant. Different features such as long term survivability of the bacterium in soil, lethal nature of the disease it causes, ability to infect many different plants, its wide geographical distribution\(^2,3\) etc. have drawn attention of several scientists across the world to engage in research related to this bacterium\(^4\).

![Fig. 1.1 Ralstonia solanacearum infectious cycle. R. solanacearum can survive in soil for a long time and invades suitable plant-host when come in contact with. It colonizes in the xylem and spread into the host and causes wilt. Thereafter it returns back to soil and resides there as saprophytes. Diagram redrawn after Genin (New Phytologist, 2010, 187, 920-928).](image-url)
Traditionally, *R. solanacearum* strains isolated from different hosts and geographical regions have been classified into five Races (Race I to Race V) on the basis of its ability to cause disease in different host plants and six biovars (Biovar I to Biovar VI) on the basis of biochemical properties such as growth on different disaccharides. Considering the difficulty in grouping diverse isolates of *R. solanacearum* strictly into different Races or Biovars, nowadays, scientists prefer the recently developed phylogenetic classification system to group different isolates into different phylotypes. Under this modern classification system, *R. solanacearum* strains are primarily grouped into four phylotypes based on the size of the 16S-23S rDNA intergenic spacer region. Phylotype of a strain and its isolation from a geographical location is related (Table 1.1, Table 1.2). For example strains exhibiting Phylotype IV pattern are all from Indonesia. The strains under a phylotype are further sub-grouped into different sequevars based on the variation in their endo-glucanase gene sequence.

### Table 1.1 Phylotypes specific multiplex PCR primers. Fegan & Prior, 2005.

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Studying virulence functions of *Ralstonia solanacearum*, the causal agent of bacterial wilt in plants

### Table 1.2 Geographical distributions of different *R. solanacearum* strains

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<td>I</td>
<td>Asia</td>
<td>GMI1000 (French Guyana), FQY_4 (China), F1C1 (India)</td>
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<td>Phylotype I is more close to Phylotype III than Phylotypes II and IV (Fig. 1.2)</td>
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<td>II</td>
<td>America</td>
<td>CFBP2957 (French West Indies), Po82 (Mexico)</td>
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<td>III</td>
<td>Africa</td>
<td>CMR15</td>
<td>13</td>
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<tr>
<td>IV</td>
<td>Indonesia</td>
<td>BDB R229 (Indonesia), <em>Ralstonia syzygii</em> R24</td>
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**Fig. 1.2 Evolutionary relationships of different phylotypes.** The phylogenetic tree constructed using *rpoB* gene which encodes for β subunit of bacterial RNA polymerase. GMI1000 which belongs to phylotype I exhibits maximum similarity with CMR15 which belongs to phylotype III. Po82 and CFBP2957 belong to phylotype II. *R. syzygii* belongs to phylotype IV. Evolutionary analyses were conducted in MEGA 6.16.

The first genome sequence of *R. solanacearum* strain was published in 2002 and was that of GMI1000 strain isolated from tomato plant.10 Till date genomes of more than ten strains have been sequenced. Different phylotypes
varies significantly with respect to their genetic compositions. Considering the immense diversity observed among different *R. solanacearum* phylotypes, scientists defines it as *R. solanacearum* species complex. Comparison of genome sequences among different strains also complies with the phylotype classification system of strains.

*R. solanacearum* genome sequence has revealed the presence of numerous virulence functions in this bacterium. The bacterium contains all the major protein secretion pathways such as type II, type III, type IV and type VI that are present in Gram –ve pathogenic bacteria. In addition to this, there are several type I and type V secretion systems in the bacterium. The bacterium has been reported to secrete more than hundred different proteins in its milieu. Various extracellular enzymes and proteins secreted through the type II protein secretion system as well as several effectors secreted through the type III protein secretion system of this bacterium have been characterized for their role in virulence and host adaptation. *R. solanacearum* genome possesses around seventy different effectors, the largest among any plant pathogenic bacteria reported till now. Effectors which are delivered to plant cell have been demonstrated to be involved in bacterial pathogenesis in plant.

As the bacterium lives in soil as well as in host plants, there is tight regulation of its gene expression for adapting to different conditions. During the infection, bacterium regulates the expression of its pathogenicity genes through sensor proteins that recognise unknown plant cell signals. Various two component regulatory systems have been characterized in this bacterium that revealed the presence of an elaborate sensory and regulatory network in this pathogen to regulate pathogenicity functions. A comparative analysis of different regulatory systems in different plant pathogenic bacteria clearly depicts the regulatory network in *R. solanacearum* is more complex in comparison to other phytopathogens.

Gene expression studies of *R. solanacearum* within the host plant have revealed that the bacterium expresses several metabolic and virulence functions quite differently in the plant than in pure culture. These findings have
given an interesting indication of sucrose availability to the bacterium inside the plant xylem.

Very recently, some exciting findings have come-up from experimental evolution research studies on *R. solanacearum*. In a very significant observation, *R. solanacearum* which lives inside the plant as an extracellular pathogen was shown to behave like an intracellular symbiotic bacterium after repeated passage of the bacterium carrying the symbiotic plasmid, in a legume plant. It was shown that mutation in a major transcription regulator such as *hrpG* has resulted in this transformation in the bacterium. In another interesting report from experimental evolution study, *R. solanacearum* has been shown to adapt well in a distant host (bacterium can grow asymptptomatically in these plant) such as bean. In this study the bacterium carried a mutation in the transcription regulator *efpR* gene. These findings give novel insights into the evolution in pathogen behaviour by transcription regulators. Genomics and transcriptomics analyses of different mutants arising from the experimental research will help us in understanding the evolution and adaptation to original hosts (*R. solanacearum* causes disease in these plants) as well as to distant hosts (*R. solanacearum* lives asymptptomatically in these plants).

The classical gene for gene interaction which is usually known to determine host range of a plant pathogen that infects only a limited number of host plants (e.g. phytopathogens belonging to Xanthomonads, Pseudomonads) has been found to be true only for few hosts. What determines host range of the bacterium is not well understood for different strains of this pathogen. Adhesion functions which occur unusually in large numbers in this bacterium have been hypothesized to have a role in determining the host range. These adhesion functions are yet to be characterized in this bacterium.

In spite of different advancements in research in regard to this pathogen, our understanding of the pathogen adaptability to host plants is incomplete. After infecting a plant, the bacterium spreads and colonizes the whole plant before killing the host. There are instances where the pathogen
lives within the plant even without killing it\textsuperscript{35, 37}. These instances indicate that unlike other bacterial pathogens (e.g. \textit{Xanthomonas}, \textit{Pseudomonas}) that attack the host soon after it comes in contact with, \textit{R. solanacearum} develops an intimate contact throughout its host plant before becoming aggressive. In this context, whether the gene expression of the bacterium is different at the initial infection stage in comparison to the later stages of infection is not elucidated. What limits the bacterium to the xylem tissue of the host plant only is not known. It is also not known about the distribution of the pathogen inside the host; there may be some preferred and selective niches for the pathogen to stay \textit{in planta}. It is important to understand the pathogen dynamics inside the host plant which is known so little. There are some aspects on which different labs are working around the world (Fig. 1.3).

![Fig. 1.3 Representing various aspects of \textit{Ralstonia solanacearum} research around the world.](image)

Fig. 1.3 Representing various aspects of \textit{Ralstonia solanacearum} research around the world. The above information and features of this bacterium provides many interesting avenues for scientists to do research on this bacterium. There are certain specific labs in the world internationally famous for their contribution towards the understanding of \textit{Ralstonia solanacearum} pathogenesis to different host.
1.2 Objectives

The main aim of the laboratory at Tezpur University is to get more insights into *R. solanacearum* gene functions and its dynamics specifically when the bacterium is inside the host plant. Accordingly, the assignment of this PhD research has been to standardize different techniques in *R. solanacearum* research for future use in the laboratory.

Different objectives of the study are as follows.

**Objective I:** Isolation and characterization of an Indian strain of *R. solanacearum*.
- Collection of wilted plants from the fields nearby Tezpur University
- Isolation of bacteria from the wilted plants
- Identification of *R. solanacearum* by using methods such as
  - Phenotypic studies (colony morphology on TZC plate, twitching motility, growth kinetics)
  - Molecular genetics analysis (transformation and natural competence)
  - Molecular analysis (phylogenetic analysis specific multiplex PCR, 16S-rDNA sequencing, Multi locus sequence typing of different genes of *R. solanacearum*)
  - Pathogenicity test on tomato plant

**Objective II:** Standardization of virulence assay on tomato seedlings.
- Standardization of the tomato seedling growth
- Infection of the tomato seedlings by the isolated *R. solanacearum*
- Creation of a *gus* tagged *R. solanacearum* and localization of the bacterium in tomato seedlings by GUS staining
- Creation of a *gspD* mutant of *R. solanacearum* by \(\Omega\)Sp (spectinomycin resistant cassette) insertion
- Extracellular cellulase assay and virulence assay of the *gspD* mutant
Objective III: Studying virulence phenotype of certain hemagglutinin mutants.

- Identification of the presence of the RSc0887 and RSp0540 homologs in the Indian isolate of *R. solanacearum*.
- Creation of independent Ω (antibiotic resistant cassette) insertion mutation in RSc0887 and RSp0540.
- Studying expression of RSc0887 and Rsp0540 by *lacZ* reporter gene fusion.
- Studying expression of RSc0887 and Rsp0540 by quantitative PCR.

1.3 Review of the literature

Bacterial wilt caused by *R. solanacearum* is a serious disease in tropical, sub-tropical and temperate regions of the world. In India, the disease was reported in West Bengal in banana. During the periods of 2009 and 2010, when this PhD research work was initiated, there was no availability of an authentic *R. solanacearum* strain in the laboratory, although presently there are many laboratories working on bacterial wilt. It was difficult to find out the availability of any *R. solanacearum* strain characterized at the molecular level in any Indian laboratories. But, quite recently, many publications from different laboratories in India have come up on *R. solanacearum* in India. In fact, an Indian isolate of *R. solanacearum* genome sequence report has been published recently. The sequenced strain belongs to phylotype I, which is in concordance with the phylotype classification of strain.

Artificial infection study of *R. solanacearum* is generally carried out on tomato plant or *Arabidopsis thaliana* plant. The infection study is generally performed by soil drench method (in which the bacterial suspension is poured in soil supporting the plant) or by stem inoculation method (bacterial suspension is directly injected into the stem of a plant). In both the methods, more than one month old grown tomato plants are inoculated with *R. solanacearum*. The wilting score is recorded in the scale of 0 (no wilting symptom) to 4 (completely wilted plant). Though these methods has been adopted to study virulence functions of *R. solanacearum*, prior to the infection
of the plant by *R. solanacearum*, the plant has already been inhabited by different endophytic bacteria from the soil. The role of these endophytes in bacterial wilt disease is not known.

Fig. 1.4 Various inoculation methods used for the infection study of *R. solanacearum*. Soil drench and stem pricking methods are most commonly used inoculation methods. Tissue culture method is recently standardized. Seedling infection process is very recent (including this work).

To omit the role of these endophytes and to understand the function of *R. solanacearum* response to only the plant, it is important to study infection in plants that are grown in laboratory condition\(^4^4\). In this process there will be less chances of *R. solanacearum* association with other bacteria during the infection process. This process will also provide an avenue to study *R. solanacearum* interaction with other endophytic bacteria by co-inoculation.

*R. solanacearum* causes a lethal wilt disease to the infected plant, which is the worst damage caused by any pathogenic bacteria to its host. Many virulence functions in this bacterium have been characterized including different two component regulatory systems, type II protein secretion systems and proteins secreted through it, type III protein secretion system and the effector proteins (Rips) secreted through it\(^2^1\). However, our understanding of *R. solanacearum* adhesion functions is not much: neither we understand well the
Rahul Kumar, Tezpur University, Tezpur (Assam)

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mechanism of attachment of the bacterium to different host plants nor we know the role of different adhesion functions in this bacterium during infection.

*R. solanacearum* genome sequence has revealed presence of many potential adhesion functions called hemagglutinins in this bacterium\(^{10,49}\) (Table no 1.3). Till date there is no report of the characterization of these functions in this bacterium. Hemagglutinin genes encode non-fimbrial adhesins in bacteria. The role of these proteins in different plant pathogenic bacteria has been illustrated. The first characterization of a hemagglutinin gene *hecA* (a homologue of *B. pertussis* filamentous hemagglutinin) was reported in *Erwinia chrysanthemi* (a broad host range pathogen). It has been demonstrated that HecA functions as an adhesin in *Nicotiana clevelandii*\(^ {45}\). Characterization of *hem* genes (*hxfA* and *hxfB*) in *X. fastidiosa* has been shown to be involved in virulence via formation of biofilm\(^ {46}\). It is pertinent to point here that factor(s) responsible for determining the host range of *R. solanacearum* is yet to be identified except in the case of tobacco plant, *Nicotiana spp.* which is not its natural host\(^ {47}\). The well-known gene for gene interaction is not applicable for the host resistance\(^ {36}\) to *R. solanacearum* as it is a broad host range pathogen. Due to the presence of striking number of *hem* genes, one of the speculations is that the adhesins might play an important role in determining the host range of this pathogen\(^ {10}\). The role of hemagglutinin in host range determination of *R. solanacearum* has also been further supported by the comparative genomic hybridization study on a pangenomic microarray of the GMI1000 reference strain\(^ {48}\). So, characterization of hemagglutinin functions in his bacterium will be an important finding in this bacterium.
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42. Ramesh, R., et al. Genome Sequencing of *Ralstonia solanacearum* Biovar 3, Phylotype I, Strains Rs-09-161 and Rs-10-244, Isolated from

43. Hanson, P. M., et al. Variable reaction of tomato lines to bacterial wilt evaluated at several locations in South East Asia, *Horti Sci.* 31, 143--146, 1996.


