STUDYING VIRULENCE FUNCTIONS OF *RALSTONIA SOLANACEARUM*, THE CAUSAL AGENT OF BACTERIAL WILT IN PLANTS

A thesis submitted in partial fulfillment of the requirements for award of the degree of

Doctor of Philosophy

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Dedicated to my

Mummy and Late Papa

Abstract

Ralstonia solanacearum causes a lethal bacterial wilt disease in many different plants. The bacterium has been reported from four continents such as Asia, Africa, South America and North America. The bacterium is now known as the second most plant pathogenic bacterium. The objective of this thesis is to standardize various molecular genetic techniques in the laboratory to address different questions relating to pathogenesis of the bacterium. The present thesis has four major chapters excluding the conclusion and future aspects.

In chapter 1, a brief introduction to *R. solanacearum* has been given. In this section different ongoing research activities in *R. solanacearum* has been mentioned briefly. This follows the objectives of the PhD work that has been undertaken for the study. Lastly review corresponding to the objectives, present status has been written under the section review of literature.

In chapter 2, different methodologies and strategies followed while collecting, isolating, identifying and molecular characterization of a *R. solanacearum* strain named as F1C1 from nearby Tezpur University campus, Tezpur, India have been discussed. The strain has been identified as a Phylotype I representative of *R. solanancearum.*

In chapter 3, standardization of an infection methodology to study pathogenicity due to *R. solanacearum* using tomato seedlings as host has been discussed. This methodology has important aspects with respect to reduced time, space consumption and economics. It is expected to supplement the earlier methodologies followed in several ways, ignoring the shortcomings.

In chapter 4, the characterization of two hemagglutinin adhesion functions namely RSc0887 and RSp0540, has been described. Insertion mutations in these two genes are created by antibiotic resistant Ω cassette. Expression of these two genes were studied by reporter gene fusion and quantitative PCR.

During the course of this PhD research several interesting questions relating *R. solanacearum* pathogenesis have come to our notice. These questions have been included in conclusion and future aspects sections.

Declaration

I hereby declare that ***"Studying virulence functions of Ralstonia solanacearum, the causal agent of bacterial wilt in plants"*** has been submitted to Tezpur University in the Department of Molecular Biology and Biotechnology under the School of Sciences for partial fulfillment for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology. This is an original work carried out by me. Further, I declare that no part of this work has been reproduced elsewhere for award of any other degree.

Date : 31/12/14

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All helps received by him/her from various sources have been duly acknowledged. No part of this thesis has been reproduced elsewhere for award of any other degree.

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All helps received by him/her from various sources have been duly acknowledged. No part of this thesis has been reproduced elsewhere for award of any other degree.

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CERTIFICATE OF THE EXTERNAL EXAMINER AND ODEC

This is to certify that the thesis entitled ***"Studying virulence functions of Ralstonia solanacearum, the causal agent of bacterial wilt in plants"*** submitted by Mr. Rahul Kumar to Tezpur University in the Department of Molecular Biology and Biotechnology under the School of Sciences in partial fulfillment of the requirement for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology has been examined by us on _____ and found to be satisfactory.

The Committee recommends for the award of the degree of Doctor of Philosophy.

Signature:

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External Examiner
Date:

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This thesis is the zenith of my journey in obtaining my Ph.D. The thesis has been kept on track and been seen through to completion with the support and encouragement of numerous people that include my colleagues, friends and well-wishers. At the end of my Ph.D. tenure, I would like to take the opportunity to thank all those people who made this thesis possible and unforgettable experience for me. It is a pleasant task to express my gratitude to all those who contributed in many ways. I sincerely apologize for my inability to acknowledge many of them, because of lack of space.

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LIST OF ABBREVIATIONS

Abbreviation	*Name*
µg	: Microgram
µl	: Microlitre
µM	: Micromolar
°C	: degree Celsius
Amp	: Ampicillin
BLAST	: Basic Local Alignment Search Tool
bp	: Basepair
cDNA	: Complementary DNA
CFU	: Colony Forming Units
conc.	: Concentration
C_T	: Threshold Cycle
d	: Days
DEPC	: Diethylpyrocarbonate
DMF	: Dimethylformamide
DMSO	: Dimethylsulfoxide
DNA	: Deoxy ribonucleic acid
dNTPs	: Deoxynucleotide triphosphates
DPI	: Days Post Innoculation
EDTA	: Ethylenediaminetetraacetic acid
EPS	: Exopolysaccharide
EtBr	: Ethidiumbromide
g $litre^{-1}$	: Gram per litre
Gm	: Gentamycin
h	: Hour
IPTG	: Isopropyl β-D-1-thiogalactopyranoside
ITS	: Internal Transcribed Spacer
kbp	: Kilobasepair

MCS	: Multiple Cloning Site
min	: Minute
ml	: Millilitre
mM	: Millimolar
MTCC	: Microbial Type Culture Collection
NCBI	: National Center for Biotechnology Information
NCM	: Nitrocellulose membrane
nm	: Nanometer
OD	: Optical Density
ONPG	: ortho-Nitrophenyl-β-galactoside
PBS	: Phosphate Buffer Saline
PCR	: Polymerase Chain Reaction
PSA	: Peptone Sucrose Agar
qPCR	: Quantitative PCR
Rif	: Rifampicin
RNA	: Ribonucleic Acid
rpm	: Rotation per minute
RT	: Room Temperature
sec	: Second
SDS	: Sodium Dodecyl Sulphate
SEM	: Scanning Electron Microscope
Spc	: Spectinomycin
spp.	: Species
TAE	: Tris-Cl, Acetic Acid and EDTA
TZC	: 2, 3, 5 - triphenyl-tetrazolium chloride
U/µl	: Units per Microlitre
X-gal	: 5-bromo-4-chloro-3-indolyl-β-D-galactosidase
X-gluc	: 5-Bromo-4-chloro-3-indolyl-β-D-glucuronidase

Chapter 1

Introduction and Review of literature

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].

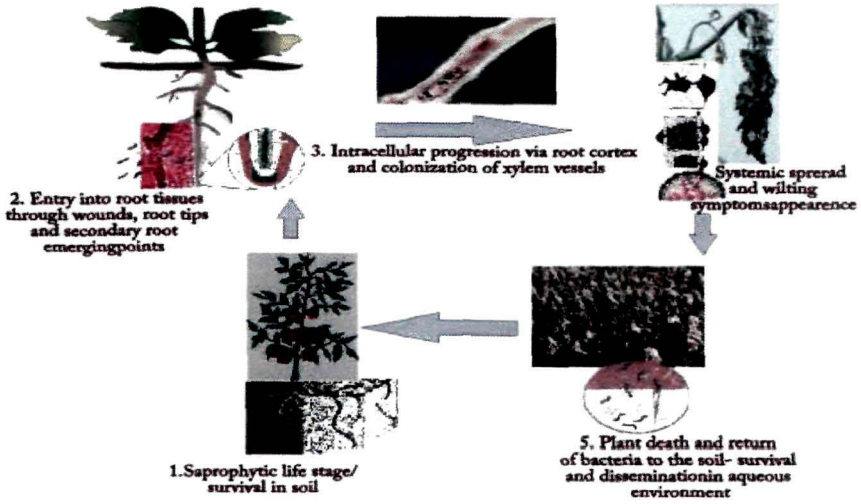

Fig. 1.1 *Ralstonia solanacearum* infectious cycle. *R. solanacearum* can survive in soil for a long time and invades suitable plant-host when come in contact with. It colonises in the xylem and spread into the host and causes wilt. Thereafter it returns back to soil and resides there as saprophytes. Diagram redrawn after Genin (New Phytologist, 2010, **187**, 920-928).

Traditionally, *R. solanacearum* strains isolated from different hosts and geographical regions have been classified into five Races (Race I to Race V)[5] 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 us growth on different disaccharides[6]. Considering the difficulty in grouping diverse isolates of *R. solanacearum* strictly into different Races or Biovars, now a days, scientists prefer the recently developed phylogenetic classification system to group different isolates into different phylotypes[7]. 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[2, 7, 8]. 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[9].

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

S. N	Primer name	Primer sequence	Specificity	Amplicon size
1	Nmult:21:1F	CGTTGATGAGGCGCGCAATTT	Phylotype I (Asia)	144
2	Nmult:21:2F	AAGTTATGGACGGTGGAAGTC	Phylotype II (America)	372
3	Nmult:23:AF	ATTACSAGAGCAATCGAAAGATT	Phylotype III (Africa)	91
4	Nmult:22:IF	ATTGCCAAGACGAGAGAAG TA	Phylotype IV (Indonesia)	213
5	Nmult:22:RR	TCGCTTGACCCTATAACGAGTA		

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

Phylotype	General occurrence	Example of some strains	Reference	Remarks
I	Asia	GMI1000(French Guyana), FQY_4 (China), F1C1 (India)	10, 11, 12	Phylotype I is more close to Phylotype III than Phylotypes II and IV (Fig. 1.2)
II	America	CFBP2957 (French West Indies), Po82 (Mexico)	13, 14	
III	Africa	CMR15	13	
IV	Indonesia	BDB R229 (Indonesia), *Ralstonia syzygii* R24	15	

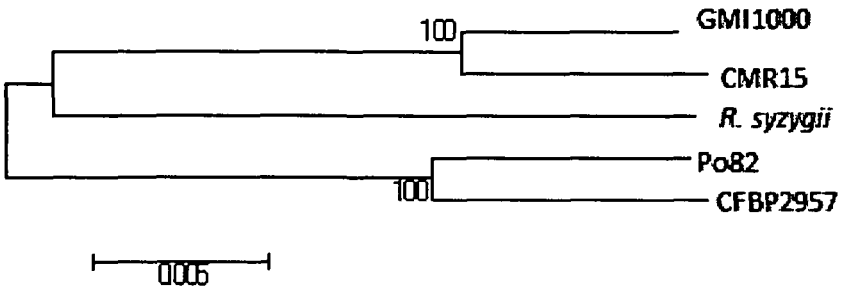

Fig. 1.2 Evolutionary relationships of different phylotypes. The phylogenetic tree constructed using *rpoB* gene which encodes for β subunit of bacterial RNA polymerase. GMI1000 which belongs to phylotype I exhibits maximum similarity with CMR15 which belongs to phylotype III. Po82 and CFBP2957 belong 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[7]. 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[17]. In addition to this, there are several type I and type V secretion systems in the bacterium[10]. The bacterium has been reported to secrete more than hundred different proteins in its milieu[11]. 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[3, 18, 19, 20]. *R. solanacearum* genome possesses around seventy different effectors, the largest among any plant pathogenic bacteria reported till now[21]. Effectors which are delivered to plant cell have been demonstrated to be involved in bacterial pathogenesis in plant[19].

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[22, 23, 24]. 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[3, 25, 26, 27, 28, 29]. 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[30].

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[31, 32]. 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[33, 34]. In another interesting report from experimental evolution study, *R solanacearum* has been shown to adapt well in a distant host (bacterium can grow asymptomatically in these plant) such as bean. In this study the bacterium carried a mutation in the transcription regulator *efpR* gene[35]. 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 asymptomatically 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[36]. 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[10].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[35, 37]. These instances indicate that unlike other bacterial pathogens (e.g. *Xanthomonas*, *Pseudomonas*) that attack the host soon after it comes in contact with, *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 *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).

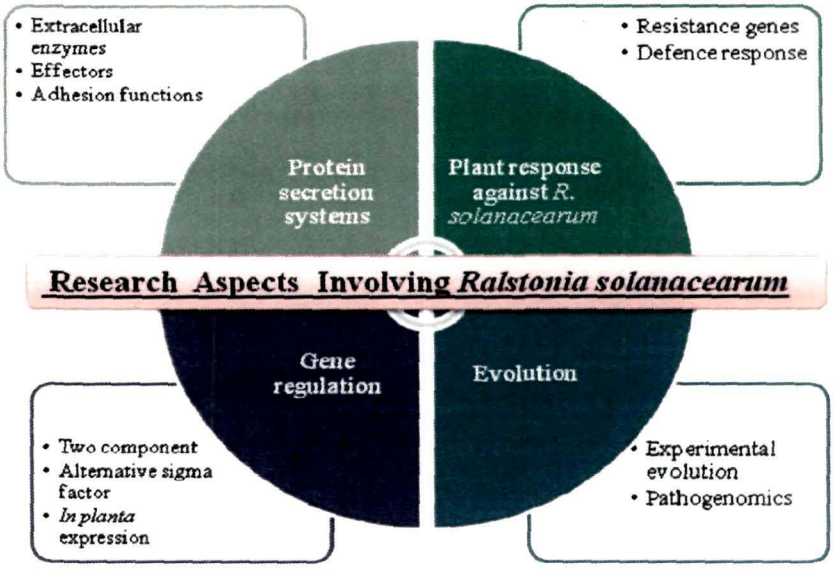

Fig. 1.3 Representing various aspects of *Ralstonia solanacearum* research around the world. The above information and features of this bacterium provides many interesting avenues for scientists to do research on this bacterium. There are certain specific labs in the world internationally famous for their contribution towards the understanding of *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 (phylotypes 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 ΩSp (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[38]. 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*[39, 40, 41, 42]. In fact, an Indian isolate of *R. solanacearum* genome sequence report has been published recently[42]. The sequenced strain belongs to phylotype I, which is in concordance with the phylotype classification of strain[7].

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) [43]. 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.

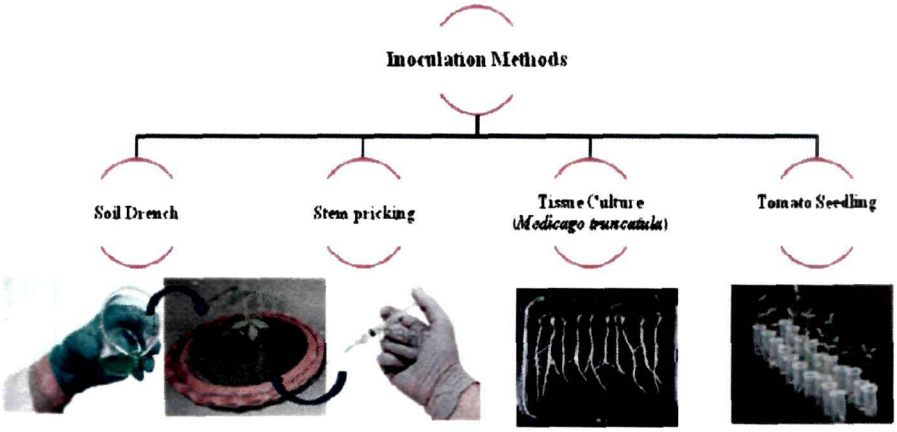

Fig. 1.4 **Various inoculation methods used for the infection study of *R. solanacearum*.** Soil drench and stem pricking methods are most commonly used inoculation methods. Tissue culture method is recently standardized. Seedling infection process is very recent (including this work).

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[44]. 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[21]. However, our understanding of *R. solanacearum* adhesion functions is not much: neither we understand well the

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.

Table 1.3 List of *hem* genes in *Ralstonia solanacearum*[10, 49]

S.N	Name	Acc.no (EMBL)	Size (bp)
1	RSc0049	CAD13577.1	1257
2	RSc0115	CAD13643.1	5019
3	RSc0127	CAD13655.1	1443
4.	RSc0887	CAD14589.1	10506
5.	RSc1495	CAD15197.1	1875
6.	RSc1775	CAD15477.1	9498
7.	RSc2796	CAD16503.1	750
8.	RSc2797	CAD16504.1	1086
9.	RSc3162	CAD16950.1	3039
10.	RSC3183	CAD16971.1	2541
11.	RSc3188	CAD16976.1	8256
12.	RSp0116	CAD17267.1	1227
13.	RSp0183	CAD17334.1	1233
14.	RSp0540	CAD17691.1	10659
15.	RSp0808	CAD17959.1	1743
16.	RSp0820	CAD17971.1	1341
17.	RSp1071	CAD18222.1	2547
18.	RSp1073	CAD18224.1	9969
19.	RSp1093	CAD18244.1	1062
20.	RSp1094	CAD18245.1	1380
21.	RSp1180	CAD18331.1	12321
22.	RSp1444	CAD18595.1	4116
23.	RSp1536	CAD18687.1	2541
24.	RSp1539	CAD18690.1	8214
25.	RSp1545	CAD18696.1	8076
26.	RSp1605	CAD18756.1	7974
27.	RSp1620	CAD18771.1	3930

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Chapter 2

Identification and establishment of genomic identity of Ralstonia solanacearum isolated from a wilted chilli plant at Tezpur, North East India

Identification and establishment of genomic identity of *Ralstonia solanacearum* isolated from a wilted chilli plant at Tezpur, North East India

2.1 Abstract

The bacterial wilt disease caused by *Ralstonia solanacearum* is an ever-increasing threat to tropical as well as temperate regions of the world. Though the disease has been reported from different parts of India, appropriate identification of the pathogen at molecular level is still incomplete. In this study we are reporting the isolation and molecular characterization of a *R. solanacearum* strain F1C1 from wilted chilli plant collected from a field nearby Tezpur University, Assam, India, using techniques such as multiplex PCR, 16S rDNA sequencing, multilocus typing, pathogenicity test, twitching motility and natural transformation. Our results suggest that F1C1 is a phylotype I strain of *R. solanacearum* species complex. Additionally, we also report presence of other bacterial species apart from *R. solanacearum* in the ooze collected from these wilted plants.

2.2 Introduction

Ralstonia solanacearum is a destructive bacterial phyto-pathogen belonging to the Class β-proteobacteria. It causes wilt disease in more than 450 plant species of 54 botanical families across the globe[1]. Owing to its wide host-range, long persistence in soil, extensive geographical distribution and profuse pathogenic nature leading to severe loss of various economically important crops, *R. solanacearum* has been ranked second among the top-ten devastating plant-pathogenic bacteria[2, 3].The pathogen is evolving rapidly and a large number of new strains have been reported quite often. Considering the genetic diversity among the strains responsible for the wilting disease in different plants, the pathogen is now termed as *R. solanacearum* species complex[4]. Traditionally this pathogen has been

classified into five races with respect to their host specificity[5, 6, 7] and six biovars according to their bio-chemical properties[6, 8, 9]. RFLP map[10, 11] have been utilized to further divide the species complex into 'Americanum' (containing biovar 1, 2 and N2 strains) and 'Asiaticum' (containing biovars 3, 4 and 5 strains) divisions respectively. Lately the bacterium has been categorized into four Phylotypes and 23 sequevars based on phylogenetic analysis of 16S-23S Internal Transcribed Spacer (ITS) region, but still lack a general agreement on sub-classification of the pathogen[12].

Since the initiation of *R. solanacearum* research in early fifties[13] several aspects relating to the pathobiology of this bacterium have been enlightened[2, 4,14,15,16,17,18,19]. The first strain of this pathogen to be sequenced was a race 1 isolate from tomato plant[20], called GMI1000, in 2002. Till date four strains of *R. solanacearum* has been sequenced with chromosome and plasmid annotation completed and another six strains with contig sequences (NCBI; http://www.ncbi.nlm.nih.gov/genome/genomes/490). Till now only one report about the genome sequence of the pathogen from Indian sub-continent is available[21]. Published literature on prevalence of *R. solanacearum* spp. complex from India is scarce and ample exploration of this important phyto-pathogen is still lacking. There is not a single strain of *R. solanacearum* available at the "Microbial Type Culture Collection and GeneBank" (MTCC) in IMTECH, Chandigarh (http://mtcc.imtech.res.in/catalogue.php), which is the national repository of microbes in India. Chattopadhyay and Mukhopadhyay (1968) reported bacterial wilt of banana (Moko disease) in West-Bengal for the first time[22]. After that no seminal work on the pathogen can be traced from this sub-continent although economic losses due this pathogen are immense. Reports on taxonomical classification of this bacterium from India are not many. Grover *et al.* (2011) have utilized short tandem repeats (STRs) at specific loci as markers to identify *R. solanacearum* isolates[23]. Recently, Chandrashekara *et al.* (2012) differentiated fifty-seven isolates of *R. solanacearum* from different wilted host plants into a

race on the basis of their pathogenicity, 16S rDNA sequence and serological tests[24]. Kumar *et al.* (2004) have performed molecular analysis of 33 strains of *R. solanacearum* obtained from Karnataka, Kerala, West Bengal and Assam by REP-PCR, ITS-PCR and RFLP-PCR dividing them into various clusters[25].

It is important to note that use of different genotype and phenotype approaches are important for correct identification of bacterium at the species level as 16S rDNA sequencing is not always the best approach for correct identification of bacterial isolates at species level[26, 27, 28]. Therefore, in this work, along with the 16S rDNA sequencing, we have utilized the widely accepted molecular method of multiplex PCR with phylotype specific primers[12] and multilocus typing to identify *R. solanacearum* from wilted host plants.

2.3 Materials and methods

2.3.1 Collection of wilted plants

The wilted plants were collected from the chilli grown fields nearby Tezpur University campus, Assam, India (26.63°N 92.8°E). The plants were collected after critically observing typical wilting symptoms. More than 10 fields were surveyed and wilted brinjal plants (egg plant), chilli plants, potato plants and tomato plants were collected (Fig. 2.1).

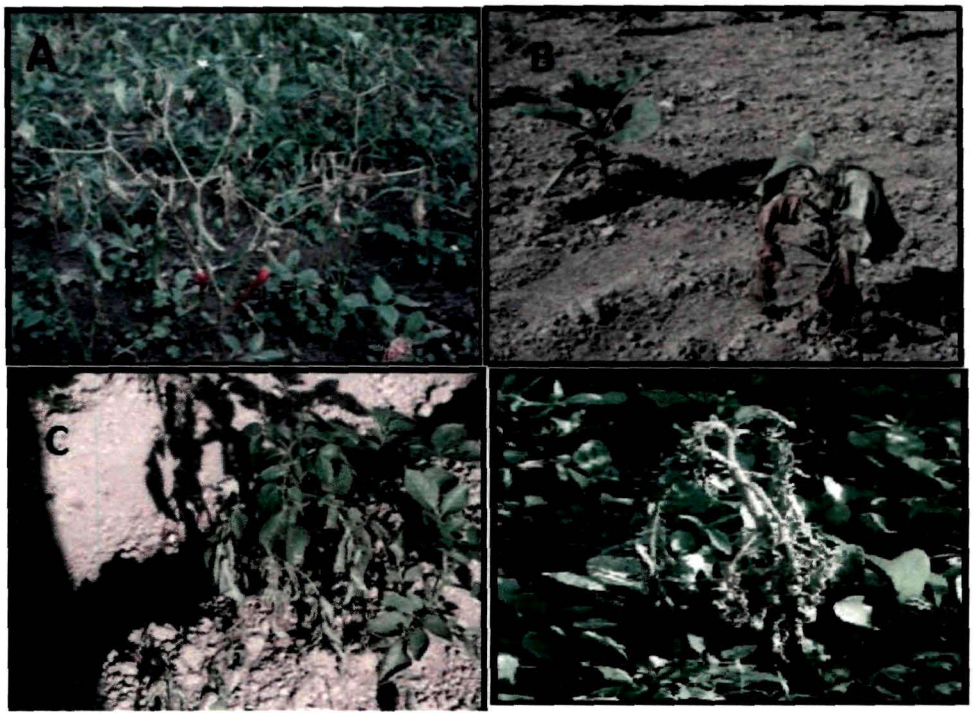

Fig. 2.1: Pictures of different wilted plants from different crop fields (A). wilted chilli plant; (B). wilted brinjal plant (egg plant) (right picture) along with a healthy brinjal plant (egg plant) (left picture) in the same field; (C). wilted potato plants along with some healthy potato plants; (D). wilted tomato plant.

2.3.2 Isolation of bacteria

Wilted plants collected were washed up with clean tap water to remove surface soil. Approximately ten centimetre stem was cut from the plant and rinsed with distilled water thrice followed by surface sterilization with 70 % ethanol. Ethanol swabbed stem portion was then rinsed with sterile water to remove ethanol from its surface. Now, this stem was cut in the middle using sterile scalpel and one of the cut ends was dipped into sterile water in a test tube.

After 10 to 15 min time interval, streams of white coloured oozes could be seen coming out of cut end. The ooze was then collected, serially diluted 10^6 folds and plated on the peptone sucrose agar (PSA) plate containing 2, 3, 5-Triphenyl

Tetrazolium Chloride (TZC). All the plates were incubated at 28°C for 48 h and were observed for the appearance of pink centred mucoid colonies.

2.3.3 Bacterial growth media

PS (1 % peptone, 1 % sucrose, 1.5 % agar in solid medium; % in weight per volume) medium was used for the culturing the bacterial isolates from wilted plants. Later on standardized BG (1 % peptone, 0.1 % yeast extract, 0.1 % casamino acid, 1.5 % agar in solid medium; % in weight per volume) medium[29] was used for culture of *R. solanacearum*. To 200 ml BG medium, 1 ml of 1 % TZC (autoclaved separately) and 5 ml of 20 % glucose (autoclaved separately) were added for observing *R. solanacearum* pink centred colony morphology. All the chemicals and growth media components were obtained from HiMedia (Mumbai, India) except casamino acid (SRL, Mumbai, India). For selection of *R. solanacearum* transformants, 50 µg/ml spectinomycin (HiMedia, Mumbai, India) conc. was used in media.

2.3.4 Twitching motility study

For observing twitching motility, F1C1 was streaked in quadrant to get the decreased concentration of the bacterium on solid BG medium. After overnight incubation (18 – 24 h) the plates were observed under the compound microscope with 4X objective. At the edges of the bacterial streaking finger like projection of bacterial growth which is a surface translocation of cells was observed. The twitching motility ceases in older colonies.

2.3.5 Pathogenicity assay on tomato plant

The bacterial isolate was checked for degree of infectivity on tomato plants (PUSA RUBY variety) grown in earthen pots. The plantlets were one month old after seedlings were planted separately. For inoculation, bacteria were grown in PS medium at 28°C for 48 h. 1 ml of this culture was pelleted down; pellet was resuspended in 1 ml sterile water and mixed by gentle pipetting. A sterile syringe needle was dipped into this culture and was used to prick the stem of tomato plants, just above the cotyledon leaves. A set of 20 plants was taken as negative control and 40 plants were taken for bacterial inoculation. Control plants were inoculated by stem pricking with sterile needle dipped in sterile water. Wilting score was done from the day when first wilting symptom was observed.

2.3.6 Polymerase chain reaction

2.3.6.1 Phylotype specific multiplex PCR

As described by Fegan and Prior (2005)[12] multiplex PCR was performed using five different phylotype specific primers:

(i) Nmult: 21:1F: CGTTGATGAGGCGCGCAATTT;

(ii) Nmult: 21:2F: AAGTTATGGACGGTGGAAGTC;

(iii) Nmult: 23: AF: ATTACSAGAGCAATCGAAAGATT;

(iv) Nmult:22:InF: TTGCCAAGACGAGAGAAGTA;

(v) Nmult: 22:RR: TCGCTTGACCCTATAACGAGTA.

Each PCR reaction was set in 15 µl reaction volume consisting of 1.5 µl of 10X Taq buffer (1.5 µl of 15 mM $MgCl_2$ was added separately to the reaction mixture), 1.5 µl of 2 mM dNTP mix, 0.2 µl of Taq polymerase (5 U/µl), 1 µl of 10 µM primer (Sigma Aldrich, India) and finally the volume was adjusted to 15 µl

with sterile de-ionized water. To the above reaction mixture, 1 µl of bacterial suspension was added as template (bacterial suspension was obtained by suspending single bacterial colony in 95 µl water followed by addition of 5 µl of 200 mM NaOH and incubation at 95°C for 10 min). PCR parameters for DNA amplification comprised overall 35 cycles: initial heating at 96°C for 5 min, denaturation at 94°C for 15 sec, annealing at 59°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 10 min in thermal cycler (Applied Biosystems; Veriti, USA). The amplified product was analysed in 2 % agarose gel and was documented (Gel doc, UVP, USA).

2.3.6.2 16S rDNA amplification

Amplification of 16S rDNA gene was performed using 16S rDNA specific primers: 27f (5'-GAGTTTGATCMTGGCTCAG-3') and 1525r (5'-AAGGAGG TGATCCAGCC-3') [30]. PCR conditions used were- step 1: 96°C for 2 min, step 2 comprising 35 cycles of 94°C for 30 sec, 55°C for 1 min, 72°C for 1 min, step 3: 72°C for 10 min (Dr. S. Genin Labs, France). The amplified DNA product was purified using quick-spin PCR purification kit (Qiagen, Germany). The purified product was then sequenced using sequencing facility (Applied Biosystems) at Tezpur University. Sequence was finally submitted to GenBank.

2.3.6.3 Multilocus typing

Gene specific primers corresponding to loci RSc0887, RSp0540, RSp1071 and RSp1073 of *R. solanacearum* GMI1000 strain were designed to check for amplification in the specific gene sequences in F1C1. RSc0887: CGTGCTACAG GCGTCCACCG (oRK001), and GAGCGGATTGGCGCTGGTGT (oRK002), RSp0540: ATGGACAGCGCGGCCTTGAC (oRK007) and GGGCGGACACGG ACAGGTTG(oRK008); CAGCGTCAACATCGGCGGGT(oRK009), TGCCGCT CGCATTGGTCTGG (ORK010), no amplification occur using this pair (oRK009 and oRK010) of primers (Fig. 2.4), RSp1071:TCACGGATGGCGCGAAGCAG

(oRK013), and CGCCCGGCATCAAATGCATCC (oRK014); RSp1073: CGGT CAACAACAACAGCGCGTC (oRK019), and CGTGCTGTCCTTGCGCCAGTT (oRK020). Sequences were retrieved from https://iant.toulouse.inra.fr/bacteria/annotation/cgi/ralso.cgi. PCR amplification consisted of total 35 cycles: initial heating at 95°C for 5 min, denaturation at 94°C for 1 min, annealing at 58°C for 30 sec and extension at 72°C for 2 min and final extension at 72°C for 10 min in thermal cycler (Applied Biosystems, Veriti, USA).

2.3.7 Natural transformation

F1C1 competent cells were prepared as described by Plener et al. (2010)[28]. FIC1 was inoculated in BG medium and allowed to grow for 48 h. 100 µl from the grown culture was added in 10 ml minimal medium (g liter^{-1}: $FeSO_4$ -$7H_2O$, 1.25 x10^{-4}; $(NH_4)_2SO_4$, 0.5; $MgSO_4$ - $7H_2O$, 0.05; KH_2PO_4, 3.4; the pH adjusted to 7 with KOH) containing 600 µl of 60 % glycerol. As the cells grow very slowly in minimal medium, turbidity of the medium does not change significantly. After 48 h of growth, 100 µl of the culture was added with 5 µg of the plasmid pRK1001 (unpublished result; with spectinomycin resistance gene). The mixture was put on top of a nylon membrane placed over solid BG medium. The cell suspension was allowed to dry inside the flow bench. The plate was incubated for 48 h at 28°C. The grown cells from the nylon membrane were mixed in 100 µl of sterile water, which was later plated on solid BG medium containing TZC, glucose and spectinomycin.

2.4 Results

2.4.1 The ooze collected from wilted plant is a mixture of different bacteria

A typical test for bacterial wilt is the observation of whitish ooze streaming out from the cut end of the infected stem after 15-20 min of exposure to water (Fig. 2.2). As expected the wilted plants collected from the fields were tested positive for bacterial wilt. To find out bacterial presence, the whitish ooze streaming out of the cut end of the wilted plant stem was collected in a test tube.

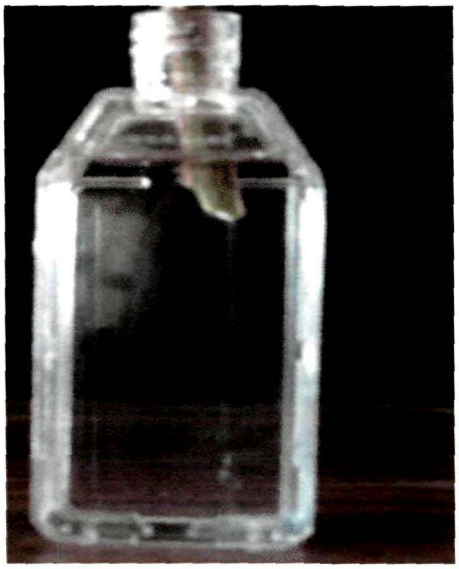

Fig. 2.2: Oozing test for isolation of bacteria from the wilted plant. The wilted plant stem section was cut and kept in sterile water. The whitish bacterial ooze was started coming out as a stream from the stem.

The ooze was then serially diluted maximum to 10^6 folds and then plated on TZC+PSA plate. Bacterial colonies were observed to appear at different intervals of incubation time such as 24, 48 and 72 h. Some colonies appeared on plate were white, dark pink and some of them were whitish with pink centre. *R. solanacearum* is known to form pink centred mucoid colony. All the bacteria with mucoid and pink centred colonies that appeared after 24 h, 48 h and 72 h after

plating were preserved. Interestingly colonies that appeared after 24 h and 48 h were having similar morphology. The bacterial colonies with pink centre and mucoid nature were further streaked on plates to get pure colony and then stored for further study. It was clear from the colony morphology and growth appearance of the colonies that the ooze contains different kinds of bacteria. All total we collected 400 bacterial isolates from different wilted plants.

2.4.2 Molecular identification of *R. solanacearum* among the bacterial isolates

To identify the *R. solanacearum* among the bacterial isolates we used the widely accepted method of multiplex PCR using phylotype specific primers[12]. In this method, a *R. solanacearum* strain belonging to any of the four phylotypes can be identified by observing the amplification of the different size phylotype specific DNA fragment. This method has been used molecular identification of many *R. solanacearum* isolates[12]. Out of total 400 isolates taken for the multiplex PCR analysis, amplification of DNA band was observed only in four isolates. All the four isolates yielded ~144 bp size DNA fragment that resembled with the standard amplification product reported from *R. solanacearum* belonging to phylotype I (Fig. 2.3).

One of the isolates from a wilted chilli plant, which we referred to as F1C1 (F1: field surveyed 1, C1: colony no 1 isolated from chilli plant), the second one is from a wilted tomato plant which we referred to as F3T23 (F3: field surveyed 3, T23: colony no 23 isolated from tomato), the third and the fourth one are from a wilted potato plant, collected from Jagatsinghpur district of Odisha state, India. To confirm the phylotype specific DNA amplification, the experiment was repeated three times with each the four strains. This result of the multiplex PCR is in agreement with the conclusion of Fegan and Prior (2005)[12] that phylotype I *R. solanacearum* strains are of Asiatic origin. All the above four isolates, exhibiting the amplification of the phylotype I specific band in multiplex PCR were found to grow slowly on PSA plates. After streaking on PSA plates, single colony appeared

only after 48 h of incubation at 28°C. This is in confirmation with the slow growth rate of *R. solanacearum*.

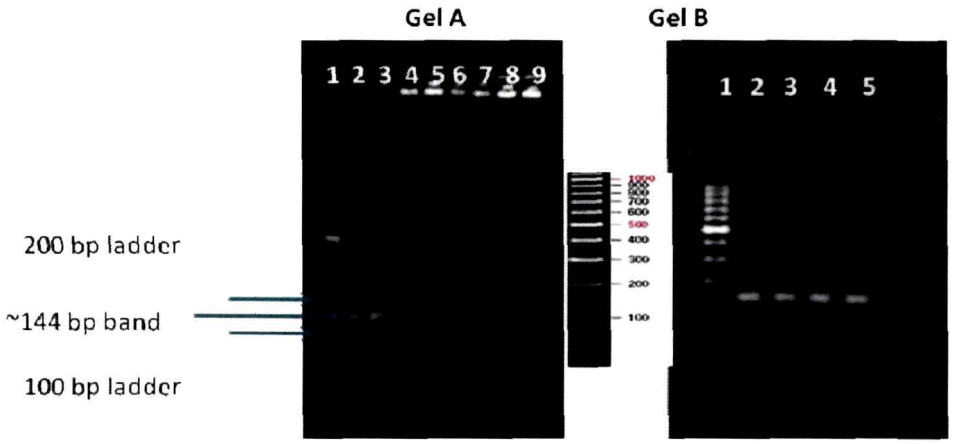

Fig. 2.3: Gel photograph of multiplex PCR of various bacterial isolates (A). In the well no. 1: gene ruler (Fermentas, UK). The size of the markers from the lower to upper end of the gel as 100 bp, 200 bp, 300 bp etc.; well no. 2 and 3: amplification of the phylotype I specific ~144 bp in F1C1; well no. 4-9: no amplification from other bacterial isolates collected from wilted plants. (B). In the well no. 1: 100 bp gene ruler (Fermentas, UK). The size of the markers from the lower to upper end of the gel as 100 bp, 200 bp, 300 bp etc.; well no. 2 to 5: confirmation of phylotype I specific band amplification in four bacterial isolates including F1C1 in the well no. 2.

One of the isolates from a wilted chilli plant, which we referred to as F1C1 (F1: field surveyed 1, C1: colony no 1 isolated from chilli plant), the second one is from a wilted tomato plant which we referred to as F3T23 (F3: field surveyed 3, T23: colony no 23 isolated from tomato), the third and the fourth one are from a

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Out of the four bacterial isolates, F1C1 was taken for further characterization. We amplified the 16S rDNA from F1C1 using universal primers. Partial sequence obtained from the amplified product exhibited 100 % homology to 16S rDNA of different *R. solanacearum* strains. The sequences were submitted to GenBank and the Accession No. are (i) BankIt1610759 Seq1 KC755042; (ii). BankIt1610759 Seq2 KC755043.

To further confirm F1C1 as *R. solanacearum*, we tried to partially amplify some of the potential pathogenicity genes in this bacterium. Primers were designed against the four hemagglutinin genes such as RSc0887, RSp0540, RSp1071 and RSp1073 of the GMI1000 genome. GMI1000 genome was followed for designing primers because it belongs to Phylotype I of *R. solanacearum* species complex and it was evident from the multiplex PCR that F1C1 also belongs to the same group. After the PCR with primers specific to different loci, amplified product of the desired size was observed in gel. Fig. 2.4 (lane 1-3) depicts the amplification of the expected 1.7 kb size DNA band from RSc0887 homolog from F1C1 genome. In Fig. 2.4 (lane 4-6) amplification of DNA band with expected size could not be observed.

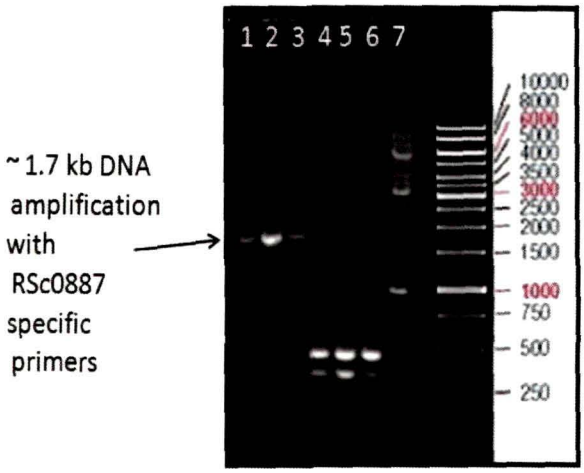

Fig. 2.4: Gel photographs demonstrating amplification of a desired size DNA in F1C1 genome using oligo designed against RSc0887 locus of GMI1000 genome. Well no. 7 has 1 kb gene ruler (Fermentas, UK). The size of the different DNA markers is given at the side. In well no 1, 2 and 3 the amplification of ~ 1.7 kb DNA from RSc0887 homologue in F1C1 is shown (oligo used were oRK001 and oRK002). In well no. 4, 5 and 6, no amplification of expected size DNA fragment using oligos oRK009 and oRK010 designed against RSp0540 locus of GMI1000. No amplification was observed in lane 4, 5, and 6 might be due to sequence difference between GMI1000 and F1C1 genomes at the primer binding region. However, RSp0540 locus presence in F1C1 has been confirmed by amplification result obtained with another set of oligos (oRK007 and oRK008) (data not shown).

For this purpose the oligo designed against RSp0540 locus of GMI1000 might have failed to pair completely with the genomic locus in F1C1 isolate. The possibility of RSp0540 absence in F1C1 was eliminated since amplification of the expected sized DNA fragment (1.7 kb) with another pair of oligos designed against RSp0540 of GMI1000 was achieved. We also got partial sequence of the two amplified products. As expected the sequence exhibited very high homology

at nucleotide level with RSc0887 and RSp0540 loci in *R. solanacearum* genome sequence.

Apart from RSc0887, RSp0540, amplification of expected sized DNA bands i.e. 1.8 kb were also observed for RSp1071 and RSp1073 homologs in F1C1. We tried to amplify long size DNA regions (1.7 kb or more) from F1C1 genome considering its future use for homologous recombination in gene insertion mutation. The experiment was repeated several times to confirm the amplification result. Although the amplified DNA regions expected from RSp1071, RSp1073 homologs are yet to be sequenced, the size of the amplified DNA strongly indicates the presence of the above GMI1000 homologues in F1C1 and a number of other regions from F1C1 genome have now been amplified in our laboratory using oligos designed against GMI1000 genome, which further indicates the presence of the homologous in F1C1 genome.

2.4.3 Twitching motility and transformation ability of F1C1

R. solanacearum has been reported to exhibit twitching motility[31]. So, we also looked for twitching motility in F1C1. F1C1 streaked plates were observed after 24 h of incubation, under the compound microscope with 4X objective. Finger like projections (Fig. 2.5B) emerging out of the streaked edges was observed on the plates, suggesting F1C1 is capable of manifesting twitching motility. Twitching motility is basically due to presence of type IV pili on Gram-negative bacterial cell envelope[32] and *R. solanacearum* demonstrate identical features like this. As a control the common laboratory strain *Escherichia coli* DH5α, and *Lysinibacillus* spp. (isolated from wilted plant; this study) were observed to be negative for twitching motility.

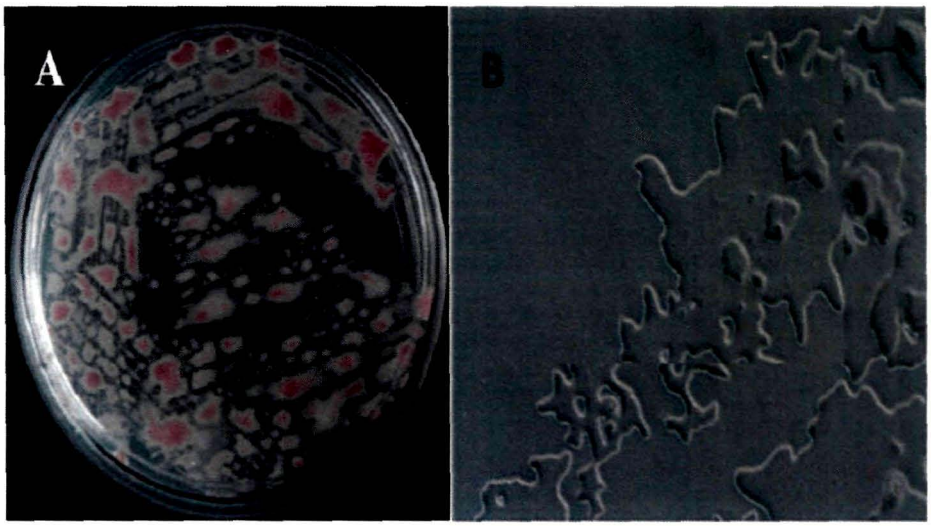

Fig. 2.5: FIC1 growth on BG plate (A). On TZC containing BG plate F1C1 forms pink centred colony having white periphery. (B). Picture depicting twitching motility in F1C1. Finger like projection (called twitching motility) from the edges of the bacterial growth are observed after 24 h streaking on BG medium. This is observed under compound microscope with 4X objective.

R. solanacearum develops natural competence for taking external DNA molecules. Therefore it is easy to knock out genes in this bacterium by homologous recombination. Twitching motility is important for natural transformation in this bacterium because mutants deficient for twitching motility are transformation deficient. As F1C1 is proficient for twitching motility, we studied natural transformation in this bacterium. We used a plasmid pRK1001 (unpublished result) to transform F1C1. The plasmid carries a partial RSc0887 gene sequence within which an omega cassette (resistant for spectinomycin) has been inserted. The linearized pRK1001 was used to naturally transform F1C1. Transformants were selected on BG containing spectinomycin. Total 120 spectinomycin resistant colonies were found out in bacteria where the plasmid was added whereas in the control in which no plasmid was added to competent F1C1

cells, not a single spectinomycin resistant colony was found. Transformation experiment was also done with other plasmid constructs (unpublished result) and the result suggested that F1C1 is efficient for natural transformation like other *R. solanacearum* strains[33].

2.4.4 Pathogenicity test of F1C1

R. solanacearum is known for its broad host range ability in causing wilting disease. F1C1 is an isolate from chilli. We tested its pathogenicity on tomato plants. Control and F1C1 inoculated plants were observed next day onwards following inoculation. Wilting symptoms were given numerical values 0 to 4 according to degree of disease phenotype observed: 0 means no wilting and 4 means complete wilting of the plant. The wilting scores are given in Fig. 2.7. On the seventh day post-inoculation, complete wilting symptoms were visible in several inoculated plants and tomato plants were seen to be dying (Fig. 2.6). In case of inoculated plants, around 25 % plants died due to wilting while some were wilted partially and others did not show any symptoms of wilting. In case of control, none of the plants exhibited wilting symptoms. The plants wilted after inoculation with F1C1 were collected and the streaming of the whitish ooze was observed that confirmed the wilting was due to bacterial infection.

Fig. 2.6 : Photographs of F1C1 inoculated wilted tomato plants. A wilted tomato plant (right side) after seven days post inoculation with F1C1. A healthy tomato plant (left side) after seven days post inoculation with sterile water. The F1C1 inoculated wilted plant was positive in the ooze test.

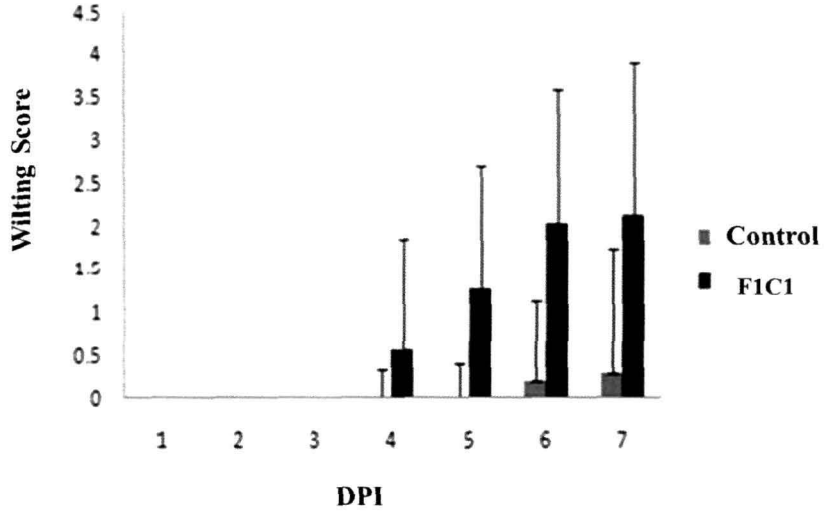

Fig. 2.7: Wilting score till seven days post inoculation Total 40 plants were inoculated with F1C1 by stem pricking and 20 plants were inoculated with sterile water by stem pricking. Wilting was scored using a scale 0.0 (for no wilting or 0 % wilting) to 4.0 (100 % wilting). First wilting symptom appeared after four days post inoculation (DPI). After seven days post inoculation while the wilting score in the F1C1 plants were 2.5, the wilting score in the water inoculated plant was only 0.15. The error bar shown is the standard deviation values.

2.4.5 Presence of other bacteria in the ooze

From the multiplex PCR analysis many of the bacteria were found to be different from *R. solanacearum*. We amplified 16S rDNA in fifteen different bacterial isolates that were looking very much like *R. solanacearum* by their colony morphology. All the bacteria were identified as *Lysinibacillus* (unpublished result). Two of the isolates were observed to promote plant growth upon inoculation (unpublished result). Other 13 bacteria are yet to be tested on plant. In a separate experiment, we observed a slow growing bacterium was inhibiting the growth of a fungus grown as a contaminant on the plate. We confirmed its antifungal activity against few fungal pathogens (unpublished result). After 16S rDNA analysis the bacterium was identified as *Alcaligenes* spp. There are many

other bacteria yet to be identified. The presence of other bacteria in the ooze along with *R. solanacearum* is intriguing. Though we had collected the ooze from the infected plant after surface sterilization, bacteria we isolated along with *R. solanacearum*, their endophytic origin cannot be claimed with certainty. The possibility of these bacterial presence on the surface of the wilted plant and have escaped the surface sterilization cannot be ruled out. In future independent inoculation experiments of these bacteria in plants as well as inoculation of these bacteria along with *R. solanacearum* in plants followed by localization study in plants will prove their association with *R. solanacearum* during infection.

2.5 Discussion

In this work, we identified a *R. solanacearum* strain from wilted chilli plant. Apart from characteristic phenotypic studies such as growth, colony phenotype on TZC medium, twitching motility, pathogenicity test on tomato plant, we used molecular techniques such as 16S rDNA sequencing, phylotype specific primer aided multiplex PCR, multi-loci typing to confirm the strain as a member of *R. solanacearum* spp. F1C1 belongs to phylotype I of *R. solanacearum* spp. complex. This finding is in agreement with the geographical distribution of the pathogen according to which phylotype I is known to be of Asiatic origin[12].

In the pathogenicity experiment we did not observe 100 % wilting in all the infected plants. This is a usual observation in *R. solanacearum* infection study (one of us has personal experience while working with *R. solanacearum* GMI1000 at LIPM, CNRS-INRA). Why some plants escape the wilting symptom (escapees) is not known. Whether the bacterium survives inside these escapees has not been investigated. Recently it has been reported that *R. solanacearum* can grow inside resistant *Arabidopsis thaliana* without causing wilting[34]. But finding the bacterium inside a susceptible host and not causing disease will be an interesting future aspect of our research.

At present complete genome sequences of only four strains of *R. solanacearum* is available in the public database. Except GMI1000, which is an isolate from French Guyana (South America), other three strains do not belong to phylotype I. Significant diversity exists among different phylotypes[35]. The whole genome sequence of F1C1 and studying its relative diversity with other sequenced strain will be interesting from the view of understanding its evolution and origin. This is also expected to illuminate different other facets of the bacterium such as intricate virulence functions, adaptive mechanisms for persistence in this particular geographical location, phylogenetic relationships with already evolved and evolving strains etc.

One of the important aspects we have observed during this isolation process which has been ignored or omitted in previous literatures is the description of persistence of several other bacteria in ooze emerging out of cut end of the wilted stem. In fact the population of *R. solanacearum* was found to be very low in the ooze collected from the cut end of the wilted stem, as only four positive isolates were found out from 400 isolates stored. There is no report available in literature regarding the quality and quantity of other bacterial association during *R. solanacearum* infection. The slow growth rate of the bacterium might be a reason of our failure to get more of it from the infected plant. A gram positive bacterium of *Lysinibacillus* species was observed to be predominant ingredient of the ooze. This bacterium appears after overnight incubation in rich medium but forms the characteristic pink centred colonies on TZC plate which resembled with that of *R. solanacearum*. As *Lysinibacillus* grows faster and the colony is mucoid in nature, the presence of this bacterium covers the whole plate and makes it difficult to identify *R. solanacearum* in the plate. Growth rate observation is critical to differentiate both the bacteria. In addition, the other simple approach might be used (which we did not try in this study) is diluting the collected ooze to 10^7, 10^8 fold before plating. This might dilute out the other bacteria leaving only the most abundant bacterium, which is likely *R. solanacearum*. As evident in earlier

literatures, we also observed the *Lysinibacillus* bacterium isolated during this study to promote plant growth upon soil inoculation as well as stem inoculation (unpublished data). Another constituent bacterium belonging to *Alcaligenes* spp. was exhibiting significant antifungal activity against few destructive fungal phytopathogens (unpublished result). Whether these other bacterial species isolated from the wilted plants remain associated with *R. solanacearum* is not known. We also do not know their exact localization in plants. The possibility that these bacteria are surface localized and have escaped the surface sterilization during ooze collection process cannot be ignored. However, isolation of *Lysinibacillus* spp. from different wilted plants, observation of its plant growth promotion activity upon independent inoculation in plants (unpublished data), and information from literature regarding its plant promoting activity, indicate towards its endophytic origin.

The environment inside plant xylem is considered as a nutritionally poor and oxygen limiting[37]. Therefore microorganisms such as *R. solanacearum* that has evolved adaptive features to survive under these circumstances were expected to outcompete other bacteria here. From the recent studies it can be understood that inside plant xylem *R. solanacearum* is in constant cross talk with plant cells[37]. A recent study on *R. solanacearum* gene expression indicates the availability of sucrose for the bacterium inside the plant xylem[37]. So the abiotic and biotic environment of xylem after and before invasion of *R. solanacearum* is going to be an interesting aspect of future research.

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Chapter 3

A simple infection assay on tomato seedlings to study pathogenecity of Ralstonia solanacearum

A simple infection assay on tomato seedlings to study pathogenicity of *Ralstonia solanacearum*

3.1 Abstract

Methods such as soil drenching and direct stem inoculation are generally used to study the virulence functions of *Ralstonia solanacearum*, the causal agent of bacterial wilt in plants. Here we are describing an easy, simple, and lesser time consuming method to study the pathogenicity of *R. solanacearum* using tomato seedlings. In this method, 6-7 days old tomato seedlings were inoculated with *R. solanacearum*. Within 8 days after inoculation, almost all the tomato seedlings were killed by the wild type *R. solanacearum* F1C1 strain. The colonization and the spreading of the bacterium throughout the dead seedlings were demonstrated by GUS staining. A *gspD* mutant of *R. solanacearum*, deficient in extracellular cellulase activity, was found to be reduced in virulence on tomato seedlings by this method. It is proposed that the seedling infection method described here might be useful in studying *in planta* behaviour of *R. solanacearum* in future.

3.2 Introduction

Ralstonia solanacearum is the causal agent of a lethal bacterial wilt disease in more than 200 plant species belonging to 50 botanical families across the globe[1]. The bacterium lives in soil as a saprophyte. In the presence of a suitable host plant, the bacterium attaches to its root and then colonizes inside it. Then it invades the xylem tissue and transmits to the aerial part of the plant employing xylem as route. Persistence of high bacterial load within the plant xylem and accumulation of copious amount of exopolysaccharides (EPS) in the xylem is assumed to be the cause of the irreversible wilting of the infected plant culminating in plant death[2]. Considering the severity of the damage rendered by

R. solanacearum, different laboratories in the world are carrying out research on this bacterium to understand its pathogenicity functions[3, 4].

In phytopathogenic bacterial virulence studies, specific bacterial pathogen is inoculated into a host plant to observe the disease pathogenesis. Freshly grown tomato plant/plantlet is a preferred host for *R. solanacearum* infection studies in most instances[5, 6]. In the existing protocol for virulence assay, one month old tomato plantlets are soil-inoculated with the bacterium and wilting symptoms, if any, are observed and recorded. In usual ground work, tomato seeds are sown to obtain seedlings that take 5-6 days to sprout. Seedlings are then transferred to pots containing soil and grown in greenhouse for about one month. Following this, plants are shifted to growth chamber where plants are inoculated with the pathogen by soil drench or stem inoculation method[5, 6]. In this approach it takes usually 40 days period to perform a single virulence assay. The infection achieved by this way is generally not axenic as soil conditions used are not devoid of other bacterial communities that can colonize the plant during its growth prior to the infection study. The association of other endophytes in turn might pose hindrance in achieving accurate result in sensitive experiments such as *in planta* expression studies[7].

Here we are describing a simple assay to study pathogenicity of *R. solanacearum* on freshly grown tomato seedlings instead of fully grown tomato plants. From seed germination to completion of the infection process, the study takes around 15 to 20 days. We believe that the infection methodology described here can be used for studying virulence functions of this bacterium.

3.3 Materials and Methods

3.3.1 Bacterial strains and growth media

Bacterial strains, plasmid and their specific characteristics used in this study are listed in Table 3.1. *R. solanacearum* was grown in BG medium[8]. The medium contains 1.0 % peptone, 0.1 % yeast extract, 0.1 % casamino acid. 1.5 % agar was added in case of solid BG agar medium (percentage in weight per volume). To 200 ml of BG medium, 1 ml of 1.0 % TZC (2, 3, 5-triphenyl tetrazolium chloride; Himedia, Mumbai, India) (autoclaved separately) and 5 ml of 20 % glucose (autoclaved separately) were added. *Escherichia coli* were grown in LB medium[9]. 1.5 % agar was added in case of LB agar medium. Concentration of antibiotics used in the above media for both *R. solanacearum* and *E. coli* were as follows: Ampicillin (Amp; 50 µg/ml), Spectinomycin (Spc; 50 µg/ml) and Rifampicin (Rif; 50 µg/ml). Stock solution of Rif was prepared in Dimethylformamide whereas that for the others was prepared in water. Antibiotic concentration in stock solution was 1000 times more than the working concentration. All antibiotics were taken from HiMedia, Mumbai, India.

Table 3.1 Bacterial strains used in this study

***Ralstonia solanacearum* strains**			
Sl no.	**Strain**	**Characteristics**	**Reference/Source**
1	F1C1	Wild type virulent *R. solanacearum* strain. It was isolated in the lab from wilted chili plant collected from a nearby field of Tezpur University, Tezpur, India	Kumar et al, 2013
2	TRS1001	Rif^r , Vir^+ strain derived from F1C1, the strain was selected as a spontaneous Rif mutant from F1C1 culture; *rif-1*	This study

3	TRS1002	*gus* +ve, Vir^{+}, Rif^{r}, Spc^{r}, this strain is derived from TRS1001 after *Tn5gusA11* insertion in an unknown locus in the genome; *rif-1zxx::Tn5gusA11*	This study
4	TRS1003	GspD deficient, Vir^{-}, Spc^{r}, extracellular cellulase deficient, this strain is derived from F1C1 by insertion of ΩSp in *gspD* gene; *gspD::*ΩSp	This study

***Escherichia coli* strains and plasmids**

Sl no.	Strain	Characteristic	Reference/Source
1	DH5α	F– Φ80*lacZ*ΔM15 Δ(*lacZ*YA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A*sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Lab collection
2	TP003	S17-1 strain carrying Tn*5gusa11* in a suicide plasmid	Lab collection
3	pGEMT	Amp^{r}; Cloning vector	Promega; Madison
4	pRS1	Amp^{r}, pGEMT plasmid containing a region of *gspD* amplified using gspDF2 and gspDR2 oligos	This study
5	pRS2	Amp^{r}, Spc^{r}, it is derived from pSR1 after insertion of ΩSp cassette in the *gspD* coding region cloned in the plasmid	This study

3.3.2 Growth of tomato seedlings

Tomato seeds (variety – S22, Evergreen seed, Bangalore, India) were put on a wet tissue paper bed in a plastic tray (Fig. 3.1). The tray was covered-up with transparent plastic sheet to maintain humidity inside at the same time facilitating the availability of light for seed germination. Seedling started appearing from 3rd day onwards and the plastic sheet was removed by the 4th day. The growth of the seedlings attained 4-5 cm in height with two cotyledon leaves on day 6th. The seedlings were ready for the infection study.

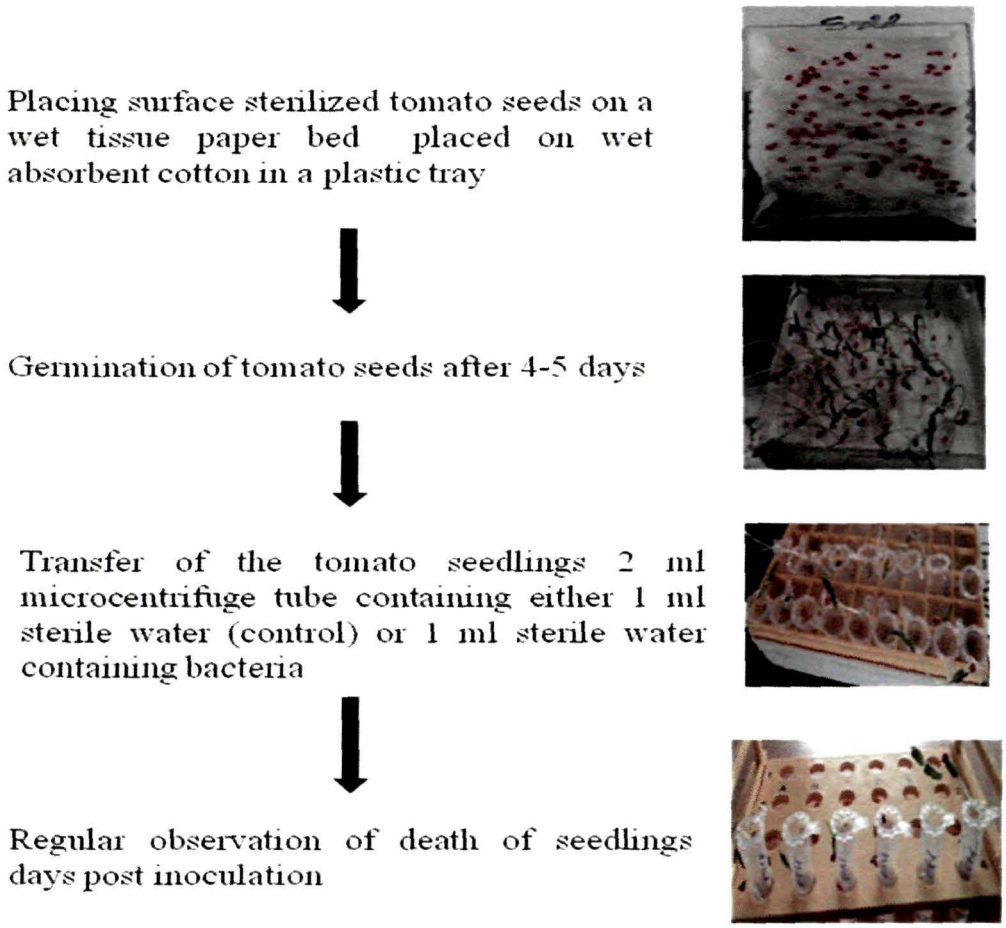

Fig. 3.1 A schematic flow diagram of the seedling assay

3.3.3 Infection assay on the seedlings to study pathogenicity of *R. solanacearum*

The 6-7 days old tender tomato seedlings were picked out gently from the tissue paper bed and were transferred to 2 ml microcentrifuge tubes containing 1 ml of sterile water. The root part of the seedling remained submerged inside the water and the stem part outside (Fig. 3.1). One seedling per microcentrifuge tubes was put. For control experiment, 20 seedlings were kept in sterile water in 20 microcentrifuge tubes. In the infection study, 100 µl of *R. solanacearum* suspension (10^9 cfu per ml) was added to 1.0 ml of sterile water present in the microcentrifuge tube was added to sterile water in each microfuge tube prior to transfer of any seedling into it. The concentration of *R. solanacearum* was 10^8 cfu per ml in the microcentrifuge tube. For a single strain infection study 20 seedlings were inoculated in one experiment. The microcentrifuge tubes containing seedlings were kept at room temperature in laboratory conditions (Fig. 3.1). Observation for seedling infection was made on every day.

3.3.4 Preparation of bacterial inoculum for infection study

F1C1 inoculum for seedling infection was prepared as follows. 30 ml of saturated F1C1 culture was taken and centrifuged at 3000 rpm for 5 min. The supernatant was thrown and the pellet was suspended slowly by adding same volume of sterile water and centrifuged again at 3000 rpm for 5 min. This washing with sterile water removed residual medium and secreted exopolysaccharide (EPS). Lastly the pellet was suspended in 30 ml water. To each microcentrifuge tube containing 1 ml of sterile water, 100 µl of the bacterial suspension was added. The above procedure was also used to prepare the inoculum of *Escherichia coli* and *Klebsiella* spp. for inoculation studies.

3.3.5 Re-isolation of F1C1 from the wilted seedling

The presence of F1C1 (*gus* tagged, Rif^r and Spc^r) bacterium inside the seedling was tested by re-isolation of the F1C1 strain from the wilted seedlings. The entire wilted seedlings were taken, washed with distilled water to remove loosely adhered bacteria on its surface. Only the stem part of the seedling that was not dipped in water in the microcentrifuge tube was cut out and crushed using mortar and pestle. Plating was done on the BG medium containing Rif and Spc antibiotics after serial dilution to select F1C1 (*gus* tagged, Rif^r and Spc^r) strains. The colonies appeared on plates were further confirmed as F1C1 (*R. solanacearum*) after performing multiplex PCR using phylotype specific primers of *R. solanacearum* as well as *gus* assay against X-gluc (Himedia, Mumbai, India) substrate.

3.3.6 Creation of *gus* tagged F1C1

The *gus* gene tagging of F1C1 was done by biparental mating between the donor *Escherichia coli* TP003 strain and the recipient *R. solanacearum* TRS1001 (F1C1 Rif^r). The former carries a constitutively expressed *gus* gene in a Tn*5* transposon[10] which is a part of the suicide conjugable plasmid. This Tn*5* is a part of the mini transposons[10]. Both the donor (TP003) and the recipient Rif^r of F1C1 (Rif mutant) were grown. The biparental mating was done according to the method described before[11]. TRS1001 (F1C1 Rif^r strain) grown in BG+Rif medium was centrifuged at 3000 rpm for five min. The supernatant was removed and the pellet was resuspended in equal volume of sterile water. Bacteria were pelleted down again by repeating the centrifugation procedure. Supernatant was discarded and the pellet was suspended in one tenth original culture volume of sterile water. 50 µl of the above suspended culture was put on top of BG agar medium in a petri-dish and left under the laminar air flow for 1 h to dry up. Donor *E. coli* TP003 cells taken by the help of sterile toothpick from a grown up plate and were mixed

with F1C1 Rif^r strain. Both the donor (TP003) and the recipient (F1C1 Rif^r) were spotted separately as control. The plate containing bacteria were incubated at 28°C for 48 h. The mixed bacteria were taken out from the plate by scraping the agar surface by the help of the sterile loop or sterile toothpick and the cells were suspended in 150 µl sterile water. Suspension of bacteria was done by vortexing for 10 sec. Then the suspension was plated on BG+Rif+Spc plates. Observation of the plates was made for 2-3 days for appearance of the transconjugants. The colonies appeared on plates were checked for Ampicillin sensitivity by streaking on BG+Rif+Spc and BG+Rif+Spc+Amp plates separately. Bacteria grew only on the former and did not grow in the later plates were considered further for the GUS assay.

3.3.7 GUS assay of tagged F1C1 colonies

In a 1.5 ml microfuge tube, 120 µl of sterile water was taken and a loop full of colonies was added followed by vortexing it for 30 sec. 1 µl of X-gluc (100 mg/ml in di-methylformamide) was added to it and incubated at 37°C for 30 min. Appearance of blue color in the suspension suggested presence of *gus* positive bacteria. The F1C1 wild type exhibited *gus* negative phenotype as expected.

3.3.8 GUS staining of seedlings

The tomato seedlings were stained with the X-gluc (50 mM sodium phosphate buffer pH 7.0, 10 mM EDTA pH 8.0, 0.1 % (v/v) Triton X-100, 0.5 mg/ml X-gluc) using a modified Jefferson *et al.* protocol (1987)[12].

The seedlings were washed several times with sterile water to remove loosely attached bacteria in its root region. Then the seedlings were kept in 2 ml microfuge tube containing X-gluc buffer (prepared as mentioned above) for 24 h. After that the seedlings were washed with sterile water and again dipped in 70 % ethanol for 3-4 h to get a better visualization of the GUS staining.

3.3.9 Creation of *gspD* mutant of F1C1 (TRS1003)

Knocking out of the *gspD* (general secretory pathway D) gene homologue in F1C1 was performed by insertion of Ω cassette (Spc^r). The oligos designed against the *R. solanacearum* GMI1000 *gspD* gene were as follows: gspD2F, 5'-GAGTCGGAACCGCTGTTGAT-3'; gspD2R, 5'-GAAGCCGTAGGTGTTCGG TC–3'. The oligos were designed to amplify an internal fragment size of 1512 bp from *gspD* homologue in F1C1 (Fig. 3.2). PCR condition was as follows:

It consist of total 35 cycles: initial heating at 95°C for 5 min, denaturation at 94°C for 1 min, annealing at 54°C for 30 sec and extension at 72°C for 2 min and final extension at 72°C for 10 min in a thermal cycler (Nexus, Eppendorf, Germany).

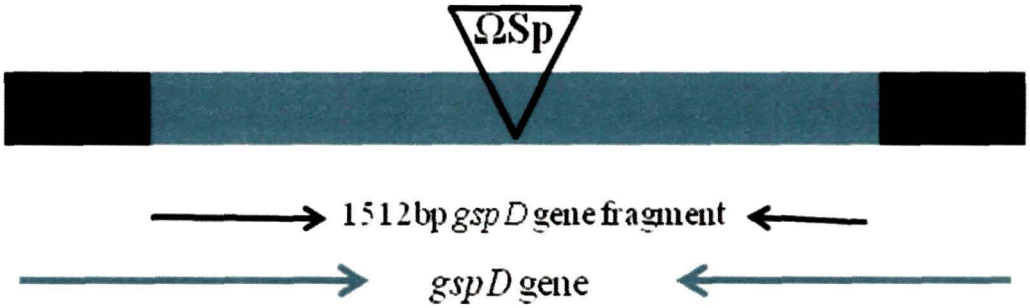

Fig. 3.2: A schematic diagram describing insertion mutation in the *gspD* homologue in F1C1 generated in this study. gspDF2 and gspDR2 are designed internal to the GMI1000 *gspD* gene . The oligos could amplify a fragment size of 1512 bp from F1C1 genome. The size is expected considering *gspD* gene of GMI1000. An ΩSp (resistant for spectinomycin antibiotic) cassette insertion at SmaI site of the amplified DNA disrupted the *gspD* coding sequence. By double homologous recombination *gspD* homolog at F1C1 genome was disrupted.

The amplicon was ligated to pGEMT plasmid vector (Promega, USA) and the ligation mixture was transformed into *E. coli* DH5α strain. The clone containing the recombinant plasmid was identified by screening for white colonies on LBA+Amp+X-gal plate, followed by colony PCR using gspD2F and gspD2R oligos and agarose gel electrophoresis of the recombinant plasmid after restriction digestion. The cloned amplicon in the recombinant plasmid was sequenced (Tezpur University sequencing facility) from both ends using the oligos gspD2F and gspD2R. Sequencing was done as per the instructions supplied by the manufacturer (Applied Biosystem, USA). The sequence obtained was analyzed by BlastN/BlastX homology[13] search and it was confirmed that the cloned amplicon was a part of the *gspD* homologue present in F1C1. The cloned DNA had a single *Sma*I restriction site in it like its homologue in GMI1000. In the *Sma*I site, the Ω cassette was inserted. Restriction enzymes and ligase used were from Fermentas (Canada) and the procedure was followed as given in the manual supplied by the manufacturer. The recombinant plasmid (pGEMT-*gspD*::ΩSp)was linearized by *Nde*I and introduced into F1C1 (*R. solanacearum*) wild type by natural transformation. Mutants were selected on Spc containing medium. Insertion mutation in *gspD* homolog in F1C1 was checked by PCR using oligos gspD2F and gspD2R. In wild type F1C1, 1512 bp was amplified while in the *gspD* mutant no amplification was observed. We further did PCR amplification using an oligo against Spc resistant gene in the Ω cassette (OSpec5-01, CGTTACCACCGCTGC GTTCGG) and the above gspD2F and gspD2R. Amplification of a DNA size 1.0 kb in the mutant using oligos gspD2F and oSpec5-01 and no amplification in the wild type strain suggested that *gspD* mutant was successfully created. The strain was further studied for extracellular cellulase activity.

3.3.10 Cellulase assay of the *gspD* mutant of F1C1

Carboxymethyl cellulose (CMC; 1 % Himedia, Mumbai, India) agar (1.5 %; Himedia, Mumbai, India) plate assay was used to study the activity of extracellular cellulase[14]. Bacterial colony from a grown up plate in BG medium was taken by toothpick and were spotted on CMC agar medium in a petriplate. The plates were incubated at 28°C for three days. The growth of bacteria and the secretion of cellulase to the exterior had occurred during the incubation. The bacterial colony was washed off with water and the medium surface was flooded with congo red 0.1 % (w/v) (Himedia, Mumbai) solution and kept for 2 h at room temperature. The congo red solution was removed and the surface was washed with water. The surface of the medium was dipped in 1 M NaCl (w/v) (Himedia, Mumbai) and the plate was kept undisturbed for 2 h. After removing the NaCl solution, the plate was observed for the presence of zone, which suggested the presence of the extracellular cellulase activity.

3.4 Results

3.4.1 F1C1 kills tomato seedlings upon inoculation

Inoculation of the 6-7 days tomato seedlings with a concentration of about ~10^8 cfu/ml of *R. solanacearum* F1C1 strain was done. Observation from the next day onwards was made for the number of seedling dying. From 4-DPI (DPI: days post inoculation) death of seedling inoculated with *R solanacearum* was recorded. On 7-DPI more than 90 % seedlings inoculated with *R. solanacearum* were found to be killed (Fig. 3.3). Whereas, percentage of killing observed in the control experiment was maximum 10 %. The result indicated that the presence of *R. solanacearum* in sterile water could accelerate the death of tomato seedlings. To understand it better, tomato seedlings were placed inside microcentrifuge tube with different loads of *R. solanacearum* such as 10^8, 10^7 and 10^6 cfu per ml. The

death % was found similar at 10^8 and 10^7 cfu per ml while the death % got delayed in the case of 10^6 cfu per ml. The result indicated that the bacterial load in sterile water was important for seedling death.

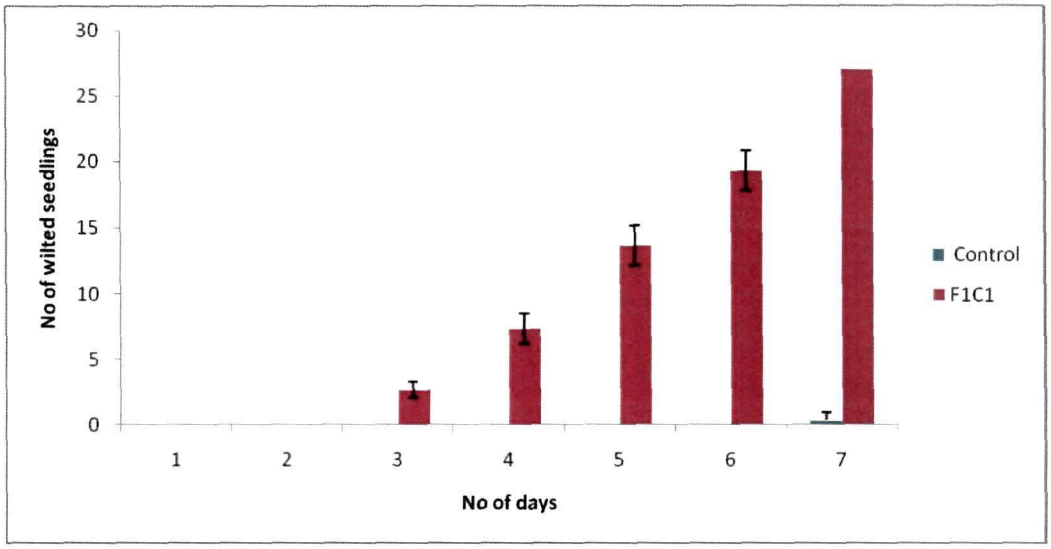

Fig. 3.3 Death of tomato seedlings under F1C1 inoculation. No. of wilted seedlings till seven days post infection with F1C1. Totally 27 seedlings were inoculated with F1C1 and 27 seedlings were inoculated with sterile water in eppendorf tube. Wilting was scored as either healthy or completely wilted like seedlings got dried completely. Initially, wilting symptoms appeared with in four days post-inoculation. After seven days all the seedlings were observed to be wilted, for the water-inoculated control seedlings only one seedling was observed to be wilted/ dried on 7^{th} day. Error bars shown are the standard deviation values.

To find out that the death of tomato seedlings was actually occurring due to *R. solanacearum* presence in the sterile water, we repeated the same inoculation process with bacteria such as *Escherichia coli* and *Klebsiella* which are not pathogenic to tomato plants. As expected the tomato seedlings were observed to be un-affected. The study demonstrated that *R. solanacearum* could cause death of tomato seedlings when inoculated in sterile water (Fig. 3.4).

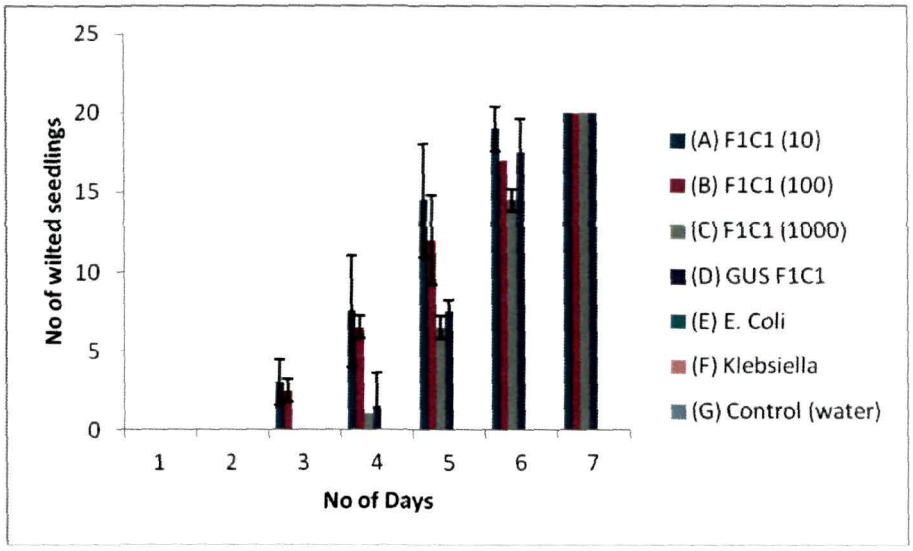

Fig. 3.4 Death of tomato seedlings under F1C1 inoculation in different concentrations. Pictures of tomato seedlings after eight days of inoculation with different bacteria. A: seedlings were kept in sterile water containing ~10^6 cfu/ml F1C1; B: seedlings were kept in sterile water containing ~10^7 cfu/ml F1C1; C: seedlings were kept in sterile water containing ~10^8 cfu/ml F1C1. D: GUS tagged F1C1 were kept in sterile water containing (~ 10^8 cfu / ml); E: seedlings kept in sterile water having *E. coli* (~ 10^8 cfu / ml); F: seedlings kept in sterile water containing *Klebsiella* strain (~ 10^8 cfu / ml); G: seedlings were kept only in sterile water. F1C1 could kill tomato seedlings at all the three concentrations. Seedlings were healthy under *E. coli* and *Klebsiella* inoculation as well as in control.

3.4.2 F1C1 colonizes the tomato seedlings

To confirm the death of seedlings occurred due to *R. solanacearum* colonization in the tomato seedling, and not due to its mere presence in the external milieu (or *ex-planta*), we inoculated the seedlings with a *gus*-marked F1C1 strain (TRS1002). Seven days old seedlings were inoculated with TRS1002.

The strain was found to be virulent like the wild type F1C1 suggesting that Tn*5* *gus* insertion in the locus had not affected the virulence of the wild type strain. The presence of bacteria in the seedling stem part was also observed by isolating *R. solanacearum* from the dead seedling by crushing only the stem part of the seedling after surface sterilization and plating it on BG medium containing antibiotics. For confirmation of *R. solanacearum*, PCR and GUS assay were carried out.

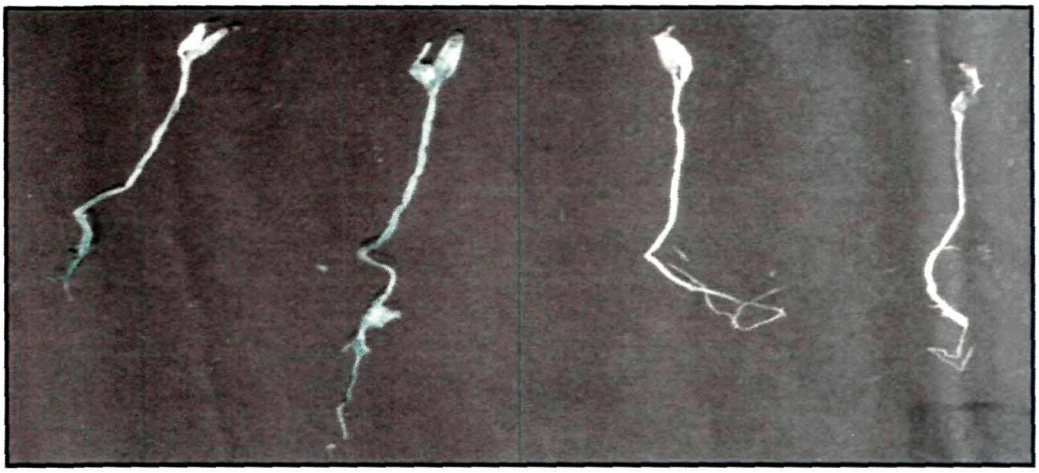

Fig. 3.5 GUS staining of tomato seedlings died of TRS1002 inoculation condition. Tomato seedling died of TRS1002 strain (*gus* marked F1C1 bacterium) inoculation, were GUS stained. These seedlings were stained blue (left side) at root, stem and cotyledon leaf regions suggesting the presence of bacteria there. The intensity of the blue staining was higher at some regions in root, stem and cotyledon leaf. The non-uniform blue staining in the seedling was observed in general in many seedlings indicating the non-uniform load of bacteria in the seedling. The control tomato seedling did not exhibit any blue color suggesting no *gus* activity in it.

We also performed GUS staining of the seedlings killed after 5 DPI as well as the control seedlings kept in sterile water to get an insight of *R. solanacearum*

colonization in the seedling. It was observed that the dead seedlings that were inoculated with TRS1002 stained blue while the control seedling appeared white (Fig. 3.5). The blue color observed in the stem and the root manifested presence of the bacteria in these parts. The detection of bacteria at the top of the seedlings suggested that bacteria were able to infect the seedlings through the roots, could colonize and also migrate to the upper part of the seedling finally causing death of the seedlings similar to that seen in a grown up infected plant. It is interesting to observe that the GUS staining was not uniform in the seedling both at the root and the stem part indicating that load of *R. solanacearum* in the different parts of the seedling might be non-uniform.

3.4.3 *gspD* mutant of F1C1 is virulent deficient on seedling

We were interested to find out if the seedling infection method could be used to study virulence function of this bacterium. GspD is an outer membrane protein that comprises of an important component of the type II protein secretion system (T2SS) in *R. solanacearum*. *gspD* mutant of different *R. solanacearum* strains were reported as virulence deficient and deficient for extracellular cellulase activity[15]. We created a *gspD* mutant of F1C1 strain by targeted gene disruption method. The *gspD* mutant of F1C1strain was found to be deficient for extracellular cellulase activity as expected (Fig. 3.6). We then studied virulence property of this strain by inoculating on tomato seedlings. At 8-DPI only 30 % seedlings inoculated with TRS1003 were found to be dead while 90 % seedling inoculated with F1C1 were dead (Fig. 3.7). This suggested that TRS003 is virulence deficient and *gspD* is required for virulence in *R. solanacearum* during seedling infection. The virulence difference between F1C1 and TRS1003 was statistically significant[16] (Fig. 3.8) ($p < 0.0001$). *gspD* encodes an outer membrane protein in type II secretion pathway. So, the *gspD* mutant's inability to cause disease might be due to its reduced ability to survive in sterile distilled water. To find out if this is the reason, we compared *gspD* mutant surviving ability with F1C1 surviving

ability in sterile water. We inoculated *gspD* mutant strain and the wild type F1C1 strain in sterile water independently as well as by mixing both the strains in sterile water. After four days and seven days, we did not find any difference between the two strains with respect to their surviving ability in sterile water. This suggested that virulence deficiency of *gspD* mutant on tomato seedling was due to their inability to infect the plant unlike the wild type.

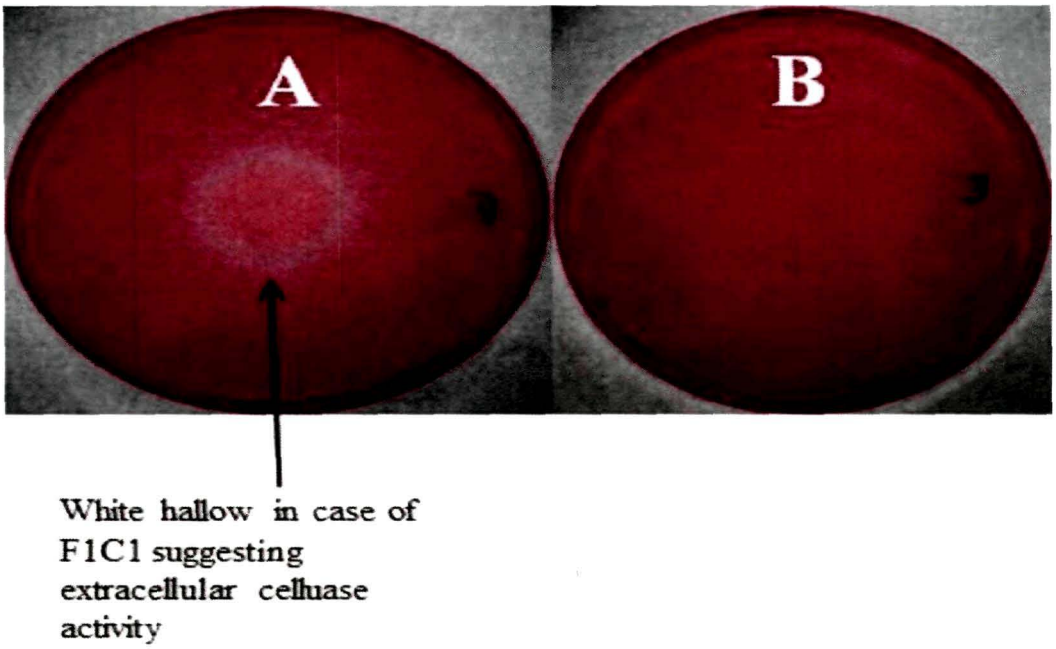

Fig. 3.6 TRS1003 (*gspD* mutant of F1C1) is deficient in extracellular cellulase activity. The wild type *R. solanacearum* (F1C1) (Plate A) and the *gspD* mutant (Plate B) were grown for three days on CMC + Agar plate. After three days of growth, the bacteria were removed by washing slowly with running tap water. The agar surface was flooded with congo red solution. Then the plates were kept undisturbed for 1-2 h. The congo red solution was removed and washed with tap water and then plate was flooded with 1 M NaCl for 2 h. Presence of a white zone at the centre was observed in case of F1C1 suggested the presence of extracellular

cellulose activity in this strain. The absence of zone in case of *gspD* mutant suggested extracellular cellulase deficiency in this strain.

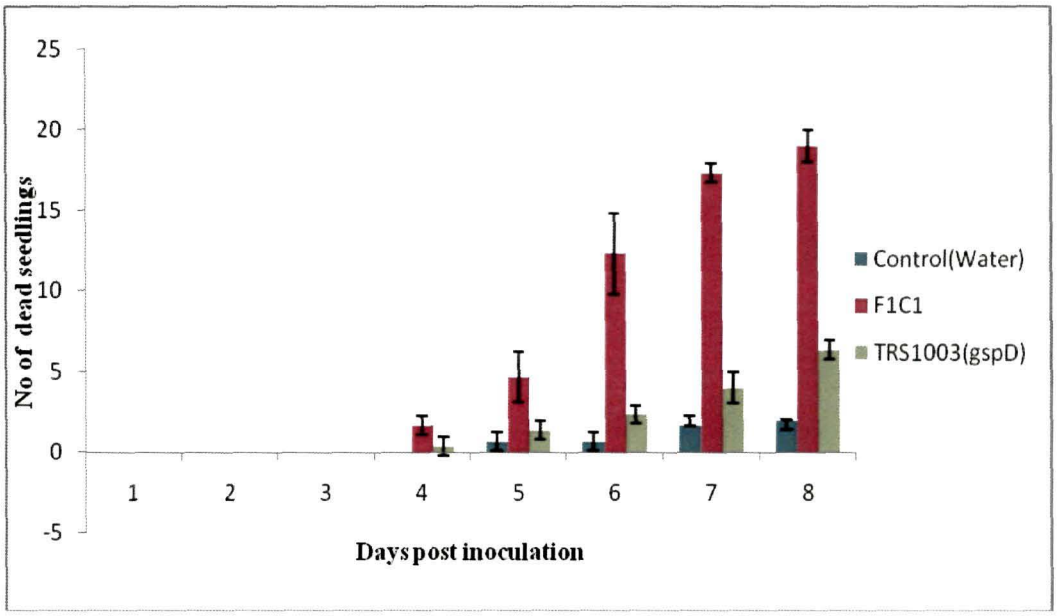

Fig. 3.7 Quantitative virulence study of F1C1 and TRS1003 (*gspD* mutant of F1C1) on tomato seedling. After eight days of inoculation, while in the case of F1C1 about 90 % seedlings died, in case of *gspD* mutant about 30 to 40 % seedlings died and in case of only water about 10 % seedlings died. The result is consistent in three independent experiment carried out. The error bar shown is the standard deviation on three experiments. The virulence difference between F1C1 and TRS1003 is statistically significant[16] (p value < 0.001; log rank test) as shown in Fig. 3.8.

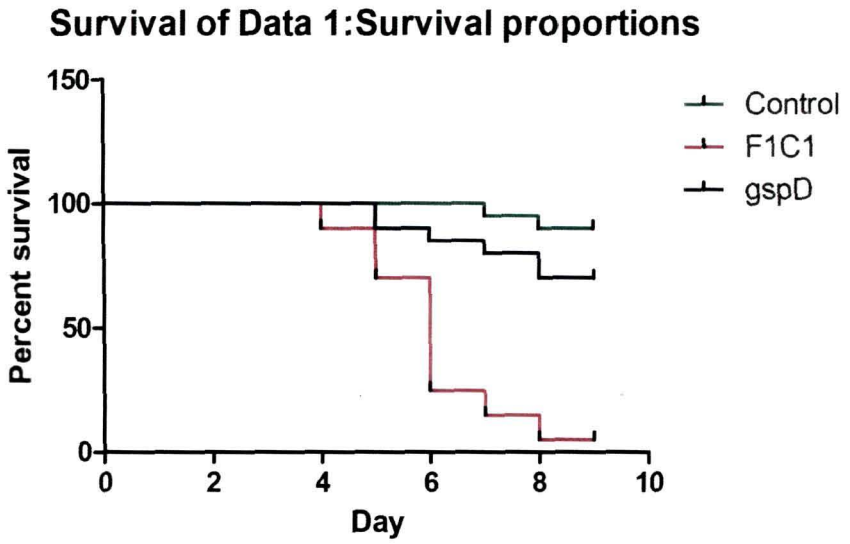

Fig. 3.8 Kaplan & Meier statistical test (log rank test) for TRS1003 (*gspD* mutant F1C1). The virulence difference between F1C1 and TRS1003 is statistically significant[16] (p value < 0.001; log rank test).

3.5 Discussion

Here we have discussed an approach to study pathogenicity of *R. solanacearum* using tomato seedling. The advantages of the approach are

- The study takes ~ 20 days starting from the seed germination to infection completion. So is less labor intensive for a single infection study.
- The infection process is sensitive to study virulence function such as *gspD*.
- The infection process avoids colonization of bacteria from soil.
- The study may be used to screen large *R. solanacearum* strains or mutants within less available space.

R. solanacearum is a vascular pathogen. The presence of *gus*-marked bacteria throughout the seedling starting from root to the cotyledon leaves

indicates that the pathogen is able to differentiate between different tissues in the seedlings. In this process known virulence functions are likely to be responsible for the infection as we understood from the virulence assay with *gspD* mutant. GUS stained seedling also indicates that the infection of the bacteriumto the seedling is a very systematic process. It is likely that the bacterium after entering into the plant near the root, it spreads to the aerial part of the seedling. During this period of spreading the bacterium is not causing severe damage to the infected seedling. Arguably it might be speculated that the bacterium is initially colonizing the seedling without killing. After spreading throughout the entire seedling the bacterium started killing the plant. A future aspect of research can be aimed at to find out gene regulation of *R. solanacearum* at different time point of infection process *in planta*.

The infection process we describe here is able to demonstrate pathogenicity due to *R. solanacearum* when there is no significant plant growth since no mineral/growth inducing factors have been added into the water. In this same condition, there is report of the bacterium's survivability without any growth[17]. In fact the seedlings looked green, turgid even after 10 days of keeping in sterile water. The *R. solanacearum* used here were earlier shown to infect the grown up tomato plants[18]. In that infection method, we had observed that all the plants inoculated were not killed. As the plant grow old, in spite of similar genotype, the plants are likely to grow differently during the growth process due to adaptation to the growth condition and different population of endophytic bacteria present in soil, which might attribute to their different response to the pathogen. In the infection method described here, the seedlings have the least chance to get exposed to the soil dwelling endophytes.

The infection process we are describing here might be helpful for study *in vivo* gene expression. There are different studies revealing genes that are expressed when the bacterium is inside the plant[7]. By now, we have understood that apart

from the pathogen, many endophytes are present inside the vascular tissues in plant. The role of these endophytes in modifying bacterial wilt symptom initiation is unknown. But, dynamics of the endophyte in the bacterial wilt can't be denied. It might be that several of these genes induced in the pathogen after entering into the host tissue is actually required for co-existence of the pathogen with the endophytes already present inside the plant xylem. To understand these, our infection procedure will be helpful. The infection process will also be helpful to study the synergistic or antagonistic effect of any endophytic bacteria in the wilt disease by doing co-inoculation. It is important to note that there are several reports[19, 20] published recently that suggest the infection of the seedling by *R. solanacearum*. However the approach we used here is to avoid contact with soil starting from seed germination to infection process completion. Therefore minimizing the possibility of colonization of any soil bacteria except the endophytes associated with the seeds. In addition in none of the previous studies neither bacteria re-isolation from the infected seedling nor its presence in the seedling by GUS staining were demonstrated.

There are likely to have several short comings in our work such as follows: Firstly, in seedling infection process the bacterium is present in liquid not in solid. We think this may not be drastically different from the natural infection process because we don't disturb the water after keeping the seedlings in it; secondly, some of the virulence functions that are expressed in a grown up tomato plant may not express in this infection process; third, the bacterium is known as vascular pathogen and in the seedlings, vascular tissues may not be fully developed. In spite of some of the potential short comings of the seedling infection process, we believe the infection procedure described here will be an additional tool for studying the pathogenicity of *Ralstonia solanacearum* in addition to the existing methods.

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Chapter 4

Characterization of filamentous hemagglutinin RSc0887 and RSp0540 homologs in Ralstonia solanacearum F1C1 strain

Characterization of filamentous hemagglutinin RSc0887 and RSp0540 homologs in *Ralstonia solanacearum* F1C1 strain

4.1 Abstract

Attachment to host is a prerequisite for pathogens to infect. Surface localized or secreted proteins, called as adhesins, play an important role in this process. *R. solanacearum* genome possesses many potential hemagglutinin adhesion functions that are yet to be characterized. RSc0887 and RSp0540 are two filamentous hemagglutinin homologs found in different *R. solanacearum* phylotype I strains. These two genes are also homologous to each other at the nucleotide level. In this study the presence of the homologs of these two genes in F1C1 strain was confirmed by PCR amplification and sequencing. To understand their role in *R. solanacearum* pathogenicity, insertion mutations were created separately in each of these two genes. Both RSc0887 and RSp0540 mutants were found to be reduced for virulence. Expression study of these two genes using *lacZ* gene fusion and quantitative PCR indicated that these two genes express very low level both in the minimal as well as in the complete medium. Our study indicates contribution of these two potential adhesion functions towards the virulence of *R. solanacearum*.

4.2 Introduction

Adhesins are surface localized proteins in bacteria that serve as the anchoring molecule and help the bacterium to adhere to different supports. On the basis of assembly mechanism and structure adhesins are classified as fimbrial and non-fimbrial adhesins[1]. Non-fimbrial adhesins are generally transported across the Gram-negative bacterial outer membrane using TypeV protein secretion system which is further divided as two partner secretion system and auto-transporter[2]. Filamentous hemagglutinins are secreted by the former type. These are encoded by usually large genes in bacteria and found in both plant and animal pathogenic

bacteria. In plant pathogenic bacteria such as *Erwinia* and *Xyllela*, filamentous hemagglutinin role in adhesion has been already demonstrated[3, 4].

R. solanacearum is a soil bacterium. In the presence of a host plant, the bacterium invades and colonizes in the xylem of it. The bacterium spreads the whole plant through xylem resulting the wilting of the plant. While growing inside the plant as well growing in soil the bacterium is likely to use different adhesion functions to tether to various supports. Genome sequence of *R. solanacearum* has revealed the presence of several potential hemagglutinin adhesion functions in this bacterium[5]. Some hemagglutinins (e.g. RSc0115; Table 1.3) are homologous to various autotransporters in bacteria while some other hemagglutinins are homologous to filamentous hemagglutinins (Fha) in bacteria. Because of their occurrence in high number in this bacterium, adhesion functions have been hypothesized as host determining factor in this bacterium[5]. However, not a single hemagglutinin adhesion function has been characterized in this bacterium till date. The reason might be scientists' greater attention towards the effectors and various regulators occurring in this bacterium.

In the present study we considered RSc0887 and RSp0450, homologs of filamentous hemagglutinin, to characterize in this bacterium. Insertion mutation in these genes indicated their role in virulence.

4.3 Material and Methods

4.3.1 Bacterial strains and growth media

Bacterial strains and plasmids used in this study are listed in Table 4.1. *R. solanacearum* strain F1C1[6] used for the present investigation was isolated from Tezpur, Assam, India. BG medium used for the growth of *R. solanacearum* was prepared as follows: Peptone- 1 %, Yeast extract- 0.1 %, Casamino Acid- 0.1 %, Agar- 1.5 %. To 200 ml BG medium, 1 ml of TZC (1 % stock, autoclaved separately) and 5 ml of glucose (20 % stock, autoclaved separately) were added for the growth of *R. solanacearum*. All the chemicals and growth media components

were procured from Himedia (Mumbai, India) except the casamino acid which was obtained from SRL (Mumbai, India).

Table 4.1 List of plasmids, *R. solanacearum* and *E. coli* strains used in this study

***Ralstonia solanacearum* strains**			
S.N	**Strain**	**Characteristics**	**Reference/ Source**
1	F1C1	Wild type virulent *R solanacearum* strain. It was isolated in the lab from wilted chili plant collected from a nearby field of Tezpur University, Tezpur, India	Kumar et. al
2	TRS1004	RSc0887 deficient, Vir^-, Spc^r, this strain is derived from F1C1 by insertion of ΩSp in RSc0887 gene; RSc0887::ΩSp	This study
3	TRS1005	RSc0887 deficient, Vir^-, Spc^r, this strain is derived from F1C1 by insertion of ΩSp in RSc0887 gene; RSc0887::ΩSp	This study
4	TRS1006	RSc0887 deficient, Vir^-, Spc^r, this strain is derived from F1C1 by insertion of ΩSp in RSc0887 gene; RSc0887::ΩSp	This study
5	TRS1007	RSc0887 deficient, Vir^-, Spc^r, this strain is derived from F1C1 by insertion of ΩSp in RSc0887 gene; RSc0887::ΩSp	This study
6	TRS1008	RSp0540 deficient, Vir^-, Spc^r, this strain is derived from F1C1 by insertion of ΩSp in RSp0540 gene; RSp0540::ΩSp	This study
7	TRS1009	RSp0540 deficient, Vir^-, Spc^r, this strain is derived from F1C1 by insertion of ΩSp in RSp0540 gene; RSp0540::ΩSp	This study

8	TRS1010	Amp^r, Gm^r, this strain is derived from F1C1 by insertion of pCZ367 in RSc0887 gene, *lacZ* insertion to study the expression of the gene, RSc0887::pCZ367	This study
9	TRS1011	Amp^r, Gm^r, this strain is derived from F1C1 by insertion of pCZ367 in RSp0540 gene; *lacZ* insertion to study the expression of the gene, RSp0540::pCZ367	This study
***E. coli* strains and Plasmids**			
S.N	**Strain**	**Characteristics**	**Reference/ Source**
1	DH5α	F– Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoAsupE44 λ– thi-1 gyrA96 relA1	Lab collection
2	pCZ367	Insertional vector with *lacZ* reporter	Lab collection
3	pGEMT	Amp^r; Cloning vector	Promega; USA
4	InsTA	Amp^r; Cloning vector	Thermo; USA
5	pRS003	Amp^r, pGEMT plasmid containing a region of RSc0887 amplified using oRK001 and oRK002 oligos	This study
6	pRS004	Amp^r, Spc^r, it is derived from pRS003 after insertion of ΩSp cassette in the RSc0887 coding region cloned in the plasmid	This study
7	pRS005	Amp^r, pGEMT plasmid containing a region of RSp0540 amplified using oRK007 and oRK008 oligos	This study
8	pRS006	Amp^r, Spc^r, it is derived from pRS005 after insertion of ΩSp cassette in the RSp0540 coding region cloned in the plasmid	This study

9	pRS007	Amp^r, InsTA plasmid containing a region of RSc0887 amplified using oRK001 and oRK002 oligos	This study
10	pRS008	Amp^r, Gm^r, it is created after taking RSc0887 amplicon by HindIII and XbaI digestion from pRS007 cloned in the pCZ367 plasmid	This study
11	pRS009	Amp^r, InsTA plasmid containing a region of RSc0887 amplified using oRK001 and oRK002 oligos	This study
12	pRS010	Amp^r, Gm^r, it is created after taking RSp0540 amplicon by HindIII and XbaI digestion from pRS007 cloned in the pCZ367 plasmid	This study

4.3.2 Identification of the presence of RSc0887 and RSp0540 homologs in F1C1

The gene specific primers for RSc0887 and RSp0540 were designed using GMI1000 as reference (https://iant.toulouse.inra.fr/bacteria/annotation/cgi/ralso.cgi.). Two sets of primers were designed. PCR amplification was performed using F1C1 genomic DNA in 15 µl reaction volume. Each PCR reaction was set in 15 µl reaction volume consisting of 1.5 µl of 10X *Taq* buffer (1.5 µl of 15 mM $MgCl_2$ was added separately to the reaction mixture), 1.5 µl of 2 mM dNTP mix, 0.2 µl of *Taq* polymerase (5 U/µl), 1 µl of 10 µM primer (Bioserve, India) and the final volume was adjusted to 15 µl with sterile de-ionized water. To the above reaction mixture, 1 µl of bacterial suspension was added as template (bacterial suspension was obtained by suspending single bacterial colony in 95 µl water followed by addition of 5 µl of 200 mM NaOH and incubation at 95°C for 10 min). PCR amplification consisted of total 35 cycles: initial heating at 95°C for 5 min, denaturation at 94°C for 1 min, annealing at 58°C for 30 sec and extension at 72°C for 2 min and final extension at 72°C for 10 min in a thermal cycler (Applied Biosystems,Veriti, USA). Following amplification the amplified DNA product

was purified using QIAquick gel extraction kit (Qiagen, Germany). The purified product was then sequenced using the sequencing facility (Applied Biosystems) at Tezpur University. The homology search was performed using the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi)[7]. The amplified product was cloned in pGEMT cloning vector and transformed in DH5α for subsequent studies.

4.3.3 Insertion mutation in RSc0887/RSp0540 homologs in F1C1

Insertion mutation in RSc0887 and RSp0540 homologs was carried out by Ω cassette[8]. Two pairs of primers were designed which amplify the fragment 1753 bp and 1747 bp with a single *Bam*HI and *XmaI*/*Sma*I restriction site respectively inside the amplified product. The fragment was cloned in pGEMT cloning vector. The ΩSp cassette[9] was inserted into the cloned fragments in the respective restriction site present inside the cloned fragment. The construct was then linearised by *Nde*I restriction enzyme[10] present in the pGEMT cloning vector and then introduced into F1C1 wild type strain by natural transformation. Spectinomycin resistant colonies appeared on the plate were checked by PCR to find out the insertion in the chromosomal copy of the respective genes.

4.3.4 Virulence assay of mutant with tomato seedlings

The virulence assay was performed with the tomato seedlings (Chapter 3 of this thesis). A bed of tissue paper was prepared and autoclaved distilled water was sprinkled in order to make it wet. Tomato seeds (variety S-22, Evergreen Seed, Bangalore) were sown on soaked tissue paper bed. Germination was evident following 7 days incubation in ambient temperature. The germinated seedlings were selected for the virulence assay. The seedlings were kept inside an eppendorf tube. The negative control was 1 ml distilled water whereas, the positive control F1C1 (*Ralstonia solanacearum*) strain was inoculated in 1 ml water. The F1C1 *hem* mutants (RSc0887::ΩSp and RSp0540::ΩSp) inoculum was prepared as described earlier. The mutant colonies were inoculated for virulence assay on

tomato seedlings. Four different colonies of RSc0887::ΩSp (TRS1004, TRS1007) and two different colonies of RSp0540::ΩSp (TRS1008, TRS1009) were inoculated in tomato seedlings for their virulence. The experiments were set up with 20 seedlings each for control and experiments. Following this the seedlings death were observed for 10 days. The experiments were carried out in triplicates and only the mean values were reported.

Table 4.2 Primers used for amplification of RSc0887/RSp0540, Spec and *lacZ* gene fragment

Gene	Primer name	Primer sequence
RSc0887	oRK001	CGTGCTACAGGCGTCCACCG
	oRK002	GAGCGGATTGGCGCTGGTGT
RSp0540	oRK007	ATGGACAGCGCGGCCTTGAC
	oRK008	GGGCGGACACGGACAGGTTG
ΩSpec	oSpec05-01	CGTTACCACCGCTGCGTTCGG
	oSpec5-02	GCCCGAGGCATAGACTGTAC
lacZ	oLacR001	AACCAGGCAAAGCGCCATTC
	oLacR002	CAAAGCGCCATTCGCCATTC

4.3.5 Cloning of RSc0887 and RSp0540 partial amplified fragment in pCZ367

The RSc0887 and RSp0540 were amplified using oRK001 and oRK002 primers (previously described PCR conditions) and cloned in InsTA cloning vector. The plasmids were isolated from the clones and digested with *Xba*I and *Hind*III. Following double restriction digestion the insert was released out. The

pCZ367 (promoter less expression vector) plasmid was isolated and digested with the *Xba*I and *Hind*III restriction enzymes. A ligation reaction was set up with the insert and pCZ367[11] linearized plasmid at 16 ^{0}C for overnight. Then the ligated product was transformed into the DH5α. The gentamycin (Gm) resistant transformants were selected.

4.3.6 Transformation of pRS008 (pCZ367::RSc0887) and pRS010 (pCZ367 ::RSp0540) into F1C1

R. solanacearum is natural competent. F1C1 was streaked on BG+TZC plate. Minimal medium were prepared (composition mentioned below) and autoclaved. In 50 ml flask 10 ml of minimal medium was taken and 600 µl of 60 % glycerol was added. A single colony of F1C1 was inoculated and allowed to grow with shaking at 200 rpm for 48 h at 28°C. Grown cells were used as the competent cells[12].

4.3.6.1 Growth in Minimal Medium

50 µl of culture grown in minimal medium was taken in two microcentrifuge tubes and mixed with 10 µl (~ 2.0 µg) of plasmid pRS008 (pCZ367::RSc0887) and pRS010 (pCZ367::RSp0540). The mixtures from the microcentrifuge tubes were placed separately on nitro cellulose (NC) membrane kept on BG medium (without glucose). Plates were incubated at 28 °C for 48 h. Thereafter bacterial cultures were taken out from the NC membrane and plated on BG + Gm (gentamycin) plate and finally incubated at 28 °C. The Gm resistant colonies were tested for the β-galactosidase activity. The *lacZ* fusion in F1C1 genome was checked by PCR using primer against *lacZ* gene and the amplicon region.

4.3.6.2 β-galactosidase activity study in bacterial colonies

In a microcentrifuge tube 120 µl of milli-Q water (Millipore, Germany) was taken and a loop full of bacteria was added. The eppendorf was tapped vigorously and 1 µl of X-Gal (50 mg/ml di-methyl formamide) was added. The mixture was vortexed and incubated at 37°C for 30 min. Blue color was observed in *lacZ* positive colonies. The positive colonies appeared were also spotted on the BG containing plate over layered with X-gal, to observe the same blue colour on plate. F1C1 and *lacZ* fusion strains (TRS1010, TRS1011) were spotted on the plate and incubated at 28°C for 48 h. Finally, the plates were observed for blue colored colonies.

4.3.6.3 β-galactosidase expression

Expression study of RSc0887 and RSp0540 was done by measuring β-galactosidase enzyme activity in TRS1010, TRS1011 strains (F1C1 strain containing a *lacZ* fusion with the promoter of the gene of interest). β-galactosidase activity was measured as µM of orthonitrophenyl-β, D-galactoside (ONPG) hydrolyzed per min per mg of cellular protein[13]. 1.5 ml of the bacterial culture was centrifuged for 2 min at 13,000 rpm. The pellet was resuspended in 750 µl of Z buffer[13] by pipetting and vortexing. The above step was repeated again in order to make the resultant volume 1.5 ml. The culture absorbance was measured at 600 nm. 750 µl of bacterial suspension was taken in an eppendorf and 100 µl of chloroform and 50 µl of 0.1 % SDS was added. The mixture was vortexed for 10 sec and incubated for 5 min at 28°C. Following this 150 µl of ONPG (4 mg per ml) was added and mixed. Once the mixture turned yellow the reaction was terminated by adding 375 µl of 1 M Na_2CO_3 Time of addition of Na_2CO_3 was recorded and then tubes were kept on ice. Finally, the tubes were centrifuged for 10 min and the absorbance was measured at 450 nm and 550 nm.

4.3.7 Quantitative PCR to study expression of RSc0887 and RSp0540 in different media

The bacterial culture was grown for 48 h in three different media i.e. BG, Minimal medium 1 and Minimal medium 2 (unpublished data) and total RNA was isolated using the TRIZOL reagent (Ambion, Life technologies, CA, USA) using standard protocol provided by manufacturer. Agarose gel was run to check the presence of intact band of RNA. RNA quantification was done using the NanoDrop (Thermo scientific, USA). Total RNA (1 µg) was reverse-transcribed into cDNA using Maxima First Strand cDNA synthesis Kit for RT-qPCR (Thermo scientific, USA) using random hexamers (using standard protocol provided by manufacturer). For the qPCR a master mix of 10 µl volume was prepared containing (2X) SYBR green - 5 µl, forward and reverse primer 1 µl each, RNAse free water - 2 µl and cDNA 1 µl. The qPCR comprised of total 40 cycles for data collection. The reactions were carried out at 95^0C for 10 min, 95^0C for 15 sec followed by 60^0C for 1 min. For determination of the melting curve the reaction was carried out at following conditions: 95^0C for 15 sec, 60^0C for 1 min followed by 95^0C for 15 sec. The *hem* (RSc0887 and RSp0540) genes expression was analyzed by quantitative real time PCR on an Applied Biosystems 7900 Real Time PCR System (Applied Biosystems, Foster City, CA) with respect to the constitutively expressing gene tuf[14] (it encodes the elongation factor Tu in bacteria). The transcripts of each target gene and of reference gene were amplified using the below mentioned primers. The threshold cycles (C_T) of RSc0887 and RSp0540 gene were averaged for triplicate reactions, and the values were normalized according to the C_T of the *tuf*. The fold change value was calculated using ΔΔCT method.

Table 4.3 List of primers used for qPCR

Gene	Primer name	Primer sequence
RSc0887	oSKR33	TCAACAACAACTACAGCGCC
	oSKR34	GTACTGATAGAGCGTCCGCC
RSp0540	oSKR37	ATACGCAGTTCAACGTTCCC
	oSKR38	TGACCTGGTTGACGATGATG
tuf	oSKR45	CGTCAAGAACATGATCACCG
	oSKR46	TGTCGCACTTGTTCAGGAAG

4.3.8 SEM analysis of wild and mutant F1C1

The wild type F1C1, the RSc0887 mutant (TRS1004) and RSp0540 mutant (TRS1008) were cultured in BG medium and the 48 h old cultures were harvested by centrifugation at 5000 rpm for 10 min. The bacterial pellet was then washed thrice with cacodylate buffer (0.2 M) (Sigma, USA) and fixed with 2.4 % glutaraldehyde (Sigma, USA) in 0.2 M cacodylate buffer. Dehydration was carried out in ethanol series with 30 min changes (30, 50, 70, 90 & 100 %). The scanning electron micrographs were acquired using scanning electron microscope (JEOL JSM-6390 LV, PTE Ltd. Singapore model). For analysis, the dehydrated bacterial biomass was sprinkled on the carbon tape and then coated with gold coat using JOEL auto fine coater (model no. JFC-1600). The morphology of the wild type F1C1 (*Ralstonia solanacearum*), TRS1004 (RSc0887 mutant) and TRS1008 (RSp0540 mutant) were observed at 20 kHz and 1 pascal pressure with a JOEL6390 scanning electron microscope and images were recorded digitally.

4.4 Result

4.4.1 RSc0887 and RSp0540 homologs in *R. solanacearum* strains

RSc0887 and RSp0540 in *R. solanacearum* GMI1000 genome are homologs of filamentous hemagglutinins found in animal and plant pathogenic bacteria. These two genes are large in size (Table 4.4) and exhibit 96 % homology with each other with query coverage of 89 %. RSc0887 is located in the chromosome and its downstream gene RSc0888 is a homolog of the outer membrane protein transporter. Usually the adhesin gene and the gene for the outer membrane transporter are present adjacent to each other in case of two partner secretion system[15] to which RSc0887 belong. RSp0540 is present in the *R. solanacearum* megaplasmid and the outer membrane transporter is not found in the downstream of this gene, which is intriguing. The strong nucleotide homology between the two genes indicates the functions are paralogous. The role of either gene duplication or horizontal gene transfer is responsible for the occurrence of the two copies of the genes. So a common transporter in outer membrane might be sufficient to transport both RSc0887 and RSp0540 across it. The BlastN analysis suggested that two copies these genes are limited to only some *R. solanacearum* strains such as GMI1000 (phylotype I), CMR15 (phylotype III), FQY4 (phylotype I), Po82 [phylotype II (B)] while in other *R. solanacearum* strains one copy of the gene is present. The occurrence of the gene in different *R. solanacearum* strains indicates its important role in this pathogen.

Table 4.4 Homology (BlastN) with RSc0887 and Rsp0540 genes

S.N	Names of representative strains/ bacteria	RSc0887 (10506 bp)		RSp0540 (10659 bp)	
		% identity	Query coverage	% identity	Query coverage
1	GMI1000 chromosome complete sequence	100	100	96	88
2	GMI1000 megaplsmid	96	89	100	100
3	FQY_4 megaplasmid, partial sequence	95	90	100	96
4	CFBP2957 plasmid RCFBPv3_mp, complete genome	91	96	90	90
5	FQY_4, complete genome	94	58	92	57
6	Po82 megaplasmid, complete sequence	91	58	89	57
7	*Ralstonia syzygii* R24, genomic contig 00001-1629	90	58	90	61
8	CMR15 plasmid CMR15_mp, complete genome	91	89	91	95
9	CMR15 chromosome, complete genome	89	22	89	23
10	PSI07 chromosome, complete genome	85	12	87	12
11	Po82, complete genome	89	9	84	12
12	*Ralstonia syzygii* R24, genomic contig 00002-1628	82	13	81	13
13	*Burkholderia gladioli* BSR3 plasmid bgla_2p, complete sequence	70	30	-	-
14	*Cupriavidus taiwanensis* str. LMG19424 chromosome 1, complete genome	76	2	72	2
15	*Ralstonia eutropha* H16 chromosome 2	73	3	73	3
16	*Burkholderia* spp. YI23 chromosome 2, complete sequence	81	1	81	1
17	*Ralstonia eutropha* JMP134 chromosome 1, complete sequence	72	2	72	2

18	*Collimonas fungivorans* Ter331, complete genome	100	0	100	0
19	*Pseudomonas parafulva* strain CRS01-1, complete genome	74	1	74	1
20	*Comamonas testosteroni* CNB-2, complete genome	82	0	82	0
21	*Azorhizobium caulinodans* ORS 571 DNA, complete genome	100	0	100	0
22	*Burkholderia thailandensis* USAMRU Malaysia #20 chromosome 1, complete sequence	-	-	97	0
23	*Burkholderia thailandensis* E444 chromosome 1, complete sequence	-	-	97	0

4.4.2 Occurrence of RSc0887 and RSp0540 homologs in F1C1 strain

Genomic DNA was isolated from the F1C1 strain of *R. solanacearum*. PCR amplification of partial region of RSc0887 homologs in F1C1 was done using oligos oRK001 and oRK002. Similarly partial region of RSp0540 homologs in F1C1 was done using oligos oRK007 and oRK008 (Table 4.2). The expected size amplicon were gel extracted and sequenced. Sequence of the amplicon revealed strong homology with the respective genes of GMI1000 (Appendix III). This suggested that the RSc0887 and RSp0540 are present in F1C1 strain, which is also a phylotype I strain.

4.4.3 Creation of chromosomal insertion mutation in RSc0887 and RSp0540 homolog of F1C1

To find out the role of RSc0887 and Rsp0540 in virulence of *R. solanacearum*, we created insertion mutation separately in both the genes (Fig. 4.1). The partial amplified fragments (Fig. 4.2) of both the genes were cloned in pGEMT (Promega, USA) TA cloning vector. The positive clones were selected by colony PCR. The plasmids (pRS003 & pRS005) were isolated and it was linearized using *Bam*H1 and *Sma*I restriction enzyme. Linear band of 4.7 kb was observed in 0.8 % gel . A ligation was set up with linearized plasmid (pRS003 & pRS005) and the ΩSp cassette having *Bam*HI and *Sma*I as overhang. Positive clones were selected on LBA+Amp+Spc plate. Transformed colonies pRS004 and pRS006 (pGEMT::RSc0887::ΩSp and pGEMT::RSp0540::ΩSp) were confirmed using digestion of the plasmid with *Bam*H1. A release of 2 kb cassette was observed in the gel (Fig. 4.3).

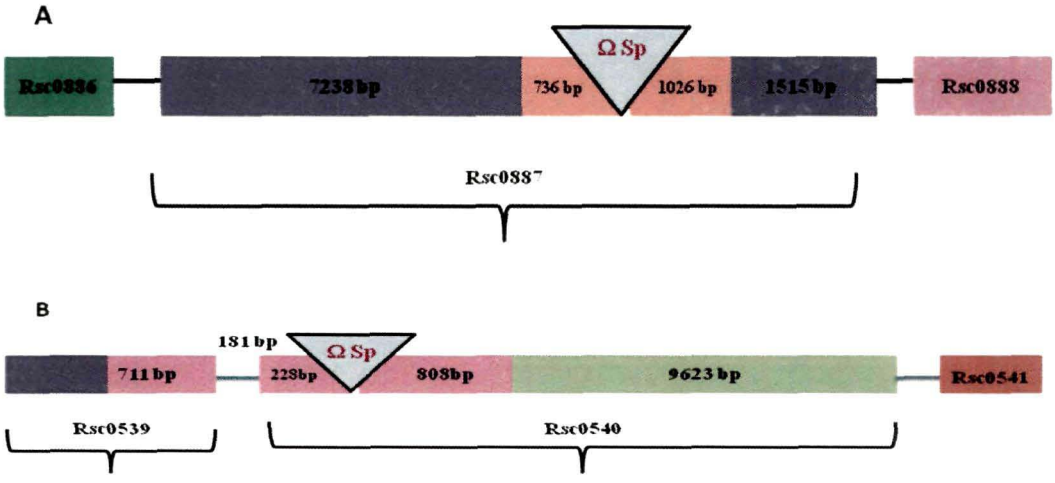


Fig. 4.1 Graphical representation of the insertion of the ΩSp in RSc0887 and RSp0540. A. Insertion of ΩSp in RSc0887, B. Insertion of ΩSp in RSp0540

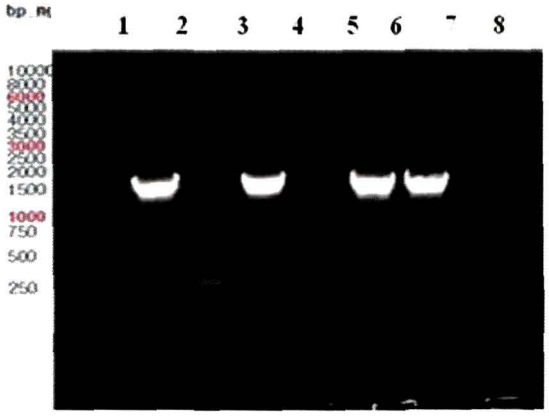
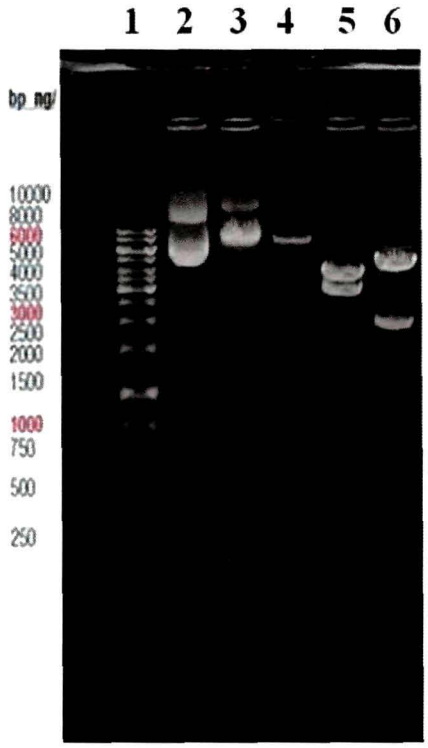


Fig. 4.2 Gel photographs demonstrating amplification of a desired size amplicon in F1C1 genome. 1.7 kb fragment amplified with oRK001 and oRK002 primers using F1C1 genomic DNA as template. Lane 1 has 1 kb gene ruler (Fermentas, UK). The size of the different DNA marker is given at the side. Lane 2 and 4 shows amplification of ~ 1.7 kb amplicon of RSc0887 homolog in F1C1 using oligos oRK001 and oRK002. Lane 6 and 7 show amplification of 1.7 kb amplicon of RSp0540 locus of in F1C1 using oligos oRK007 and oRK008.

Again, the cassette insertion was confirmed using digestion with different enzymes to make it linear, and release of the cassette, and release of the insert. The plasmid was liniearized with the *Nde*I that resulted a DNA size of 6.7 kb. With *EcoR*I digestion present on the both side of the vector the release of the insert (1.7 kb) with the cassette (2 kb) displayed a band of 3.7 kb and another band of 3 kb (which was of the vector). When the same plasmid was digested with the *Bam*HI which was present in the insert it released the cassette resulting in two bands one of the 2 kb cassette and the other of 4.7 kb comprising of the vector and the insert.



Lane 1- 1kb ladder
Lane 2- pRK002
Lane 3- pRK002 + Nde1 partial
Lane 4- pRK002 + Nde1 complete
Lane 5- pRK002 +EcoR1
Lane 6- pRK002 +BamH1

Plasmid size- ~6.7 kb
Nde1 (single cutter) linear- ~6.7
BamH1(to remove cassette)- 2 Kb + 4.7kb
EcoR1(to remove insert + cassette) – 3.7kb+ 3 kb

Fig. 4.3 Gel showing insertion of Ω cassette in pRS003. Digestion of pRS004 (pGEMT:: RSc0887::ΩSp) with different restriction enzymes to release Ω cassette. Lane 1 has 1 kb gene ruler (Fermentas, UK). Size of the marker is 250 bp, 500 bp, 750 bp, 1000 bp etc. Lane 2 has uncut plasmid, lane 3 partial digestion of pRS004 plasmid, Lane 4 digestion of pRS004 with *NdeI* shows ~6.7 kb linear plasmid, lane 5: digestion of pRS004 with *Eco*RI gives two band of 3.7 kb (1.7 kb RSc0887:: ΩSp) and 3 kb (pGEMT) vector, lane 6: pRS004 digestion with *Bam*HI shows 4.7 kb band (pRS003) and 1.7 kb RSc0887 amplicon.

The plasmid with the insert and the cassette were transformed. Homologus recombination occurred in F1C1. Many of Spc resistant colonies were observed, in which the wild type fragment from RSc0887 and RSp0540 could not be amplified thereby indicating the ΩSp insertion in RSc0887 and RSp0540. The insertion was confirmed with the PCR study using one RSc0887 primer and other primer

designed specific to ΩSp cassette (OSpec05-01) Table 4.1 The bacterium appeared was also checked by multiplex PCR to rule out any contamination.

4.4.4 RSc0887 and RSp0540 mutants are virulence deficient

Four insertion mutants of RSc0887 (TRS1004-TRS1007) and two insertion mutants of RSp0540 (TRS1008, TRS1009) were inoculated on tomato seedlings to study their virulence. The virulence was scored as 0.0 for seedling survival and 1.0 for seedling death. The result is shown in Fig. 4.4. From day 4 onwards seedlings inoculated with wild type F1C1 started dying whereas in control where there were no bacteria, seedlings were normal. After 8 days of inoculation almost all the seedlings inoculated with the wild type F1C1 died whereas in control the plants were normal. In comparison to that, RSc0887 mutants were very much reduced for virulence. Maximum three seedlings were dead out of 20 seedlings inoculated by different strains TRS10004 –TRS1007. This indicated that RSc0887 were severely deficient for virulence. RSp0540 mutants (TRS1008, TRS1009) though were found virulence deficiency but the magnitude of virulence deficiency was less severe in comparison to RSc0887 mutants.

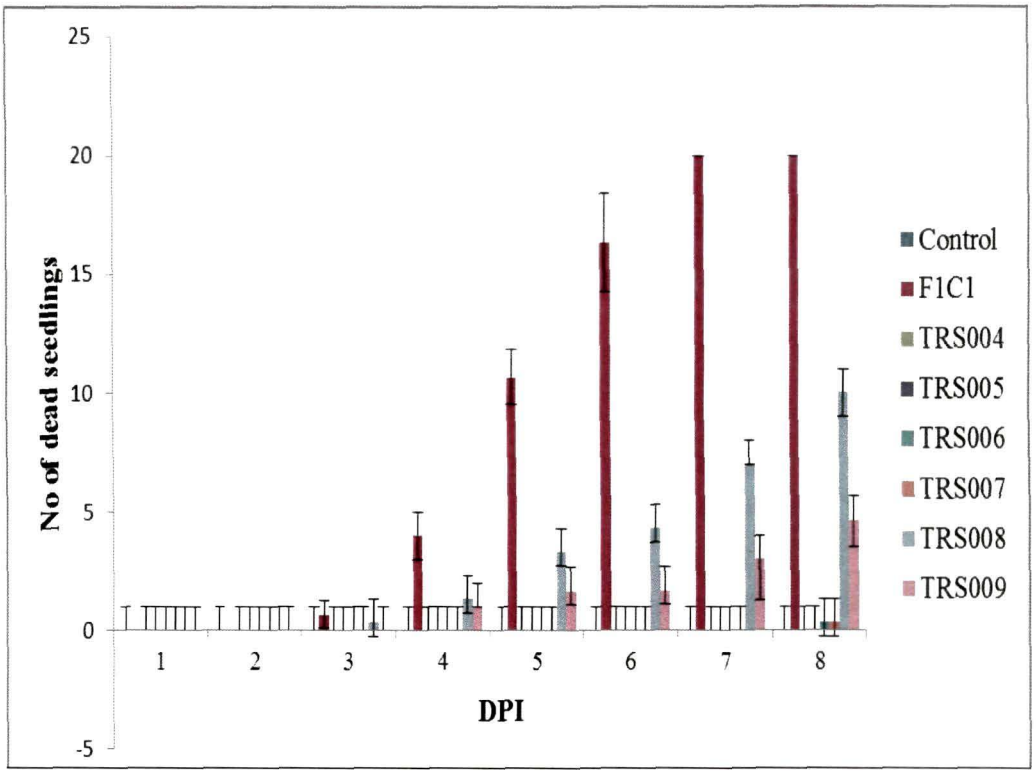


Fig. 4.4 Virulence assay of RSc0887 and RSp0540 mutants using tomato seedling. In the control only water was added and for positive control F1C1 is used. Four different colony of RSc0887 and two of RSp0540 were taken for study and symptoms were observed upto 8 days post inoculation. TRS004-TRS007 shows virulence deficient but TRS008 and TRS009 shows reduced virulence

4.4.5 Expression study of RSc0887 and RSp0540 using *lacZ* reporter gene fusion assay

The partial fragment amplified from the RSc0887 and RSp0540 were successfully cloned in pCZ367 (Fig. 4.5). The recombinant plasmids pRS008 (RSc0887) and pRS0010 (RSp0540) were introduced into wild type F1C1 by natural transformation. Gentamycin resistant colonies selected were checked for blue colony formation on X-gal containing plate (Fig. 4.6). Gene specific primer and *lacZ* primer were used to know the insertion was in F1C1 genome.

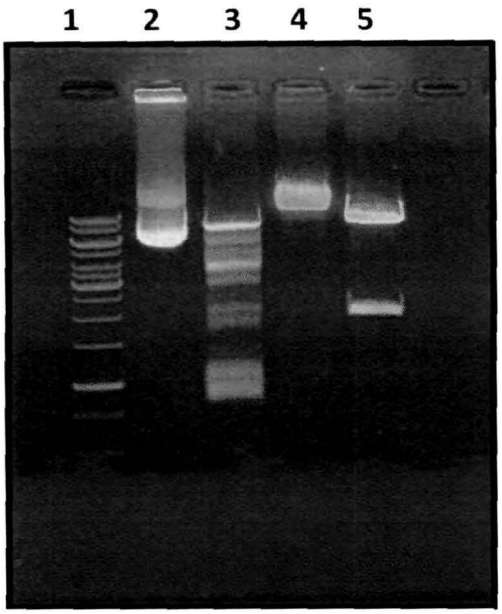


Fig. 4.5 pRK006 (RSc0887::pCZ367) cloning conformation gel. The pRS008 (pCZ367::RSc0887) plasmid was disgested using different enzymes to release the insert from the pCZ367 vector. Well 1: 1 kb ladder, well 2: uncut pRS008 plamid, well 3: pRES008 digested with BamHI, well 4: pRS008 digestion with HindIII to make the plasmid linear. Well 5: pRS008 digestion with XbaI and HindIII released the insert from pCZ367

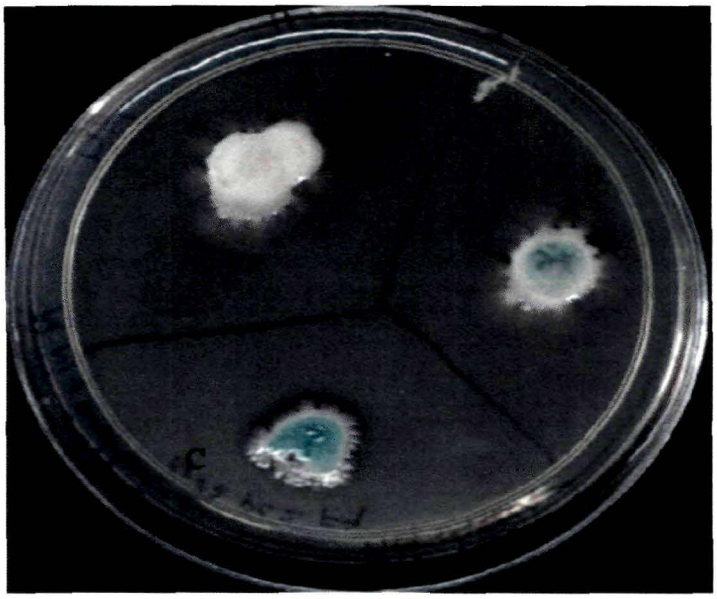


Fig. 4.6 Expression study of TRS1011 (F1C1::RSp0540::pCZ367) on X-gal plate. TRS1011 (F1C1::RSp0540::pCZ367) colony were spotted on BG+X-gal plate. A: F1C1 is spotted as control and gives white colour colony after incubation. B & C: TRS1011 strain was spotted the same plate. As pCZ367 is a promoter-less vector the *lacZ* expression is occurred after the gene specific promoter where it get inserted. The presence of blue colour in B and C clearly shows its expression.

β-galactosidase expression was quantified monitored after growing the bacteria (F1C1, TRS1010, TRs1011) in three different media such as BG medium, minimal medium and minimal medium 2. *lacZ* expression in units is given in Fig. 4.7 and 4.8. F1C1 wild type there was no activity as expected. In TRS1010 (RSc0887) the activity was very low in all the three medium[16]. In case of TRS1011, the activity was higher than the TRS1010 and also the activity was more in minimal medium than the BG. Overall from the β-galactosidase expression study indicated that both RSc0887 and RSp0540 expression was not high in all the three conditions.

Fig. 4.7 Expression assay of RSR001 (F1C1::pCZ367::RSc0887) using ONPG assay A. showing F1C1 as control does not show any colour; B. yellow colour shows the expression of the RSc0887 gene.

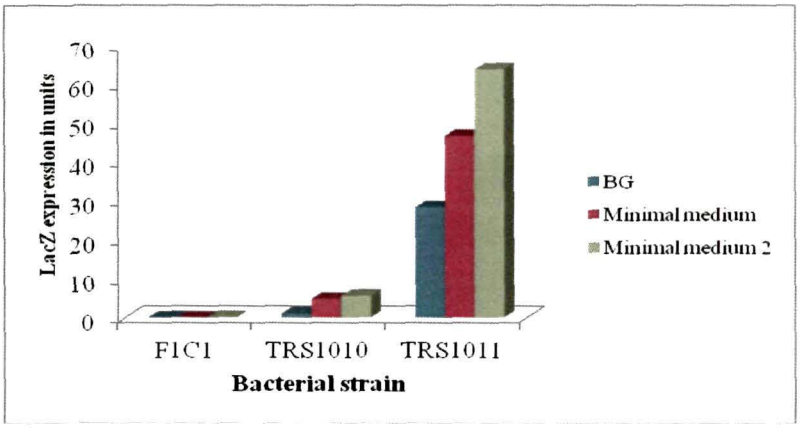


Fig. 4.8 Expression pattern of the RSc0887 and RSp0540 genes. Expression study was carried out in complete, minimal medium and minimal medium 2. The y-axis indicates β-galactosidase activity expressed in Miller units and the x-axis indicates the different conditions in which the expression study was carried out. Standard deviation was calculated from three different experiments performed independently.

4.4.6 Expression analysis of RSc0887 and RSp0540 genes by quantitative PCR

Expression of RSc0887 and RSp0540 was quantified in wild type F1C1 grown in the three different media such as BG, minimal medium, minimal medium 2 using quantitative PCR. Elongation factor Tu (*tuf*) gene was used as control in

the expression study. In BG medium expression of RSc0887 found to be lower than RSp0540. But in minimal medium the expression of RSc0887 found to be two times more than the RSp0540. In case of minimal medium 2, expressions of both the genes were found to be similar. Though there was difference in their expression pattern under different media, the expression levels were not drastically different. The RSc0887 gene possessed higher expression level in BG medium in comparison to RSp0540 (Fig. 4.9). But in the minimal medium the RSp0540 express higher than the RSc0887. But RSc0887 and RSp0540 showed similar expression level in the minimal medium 2 suggesting that both the genes were differentially regulated by the media composition.

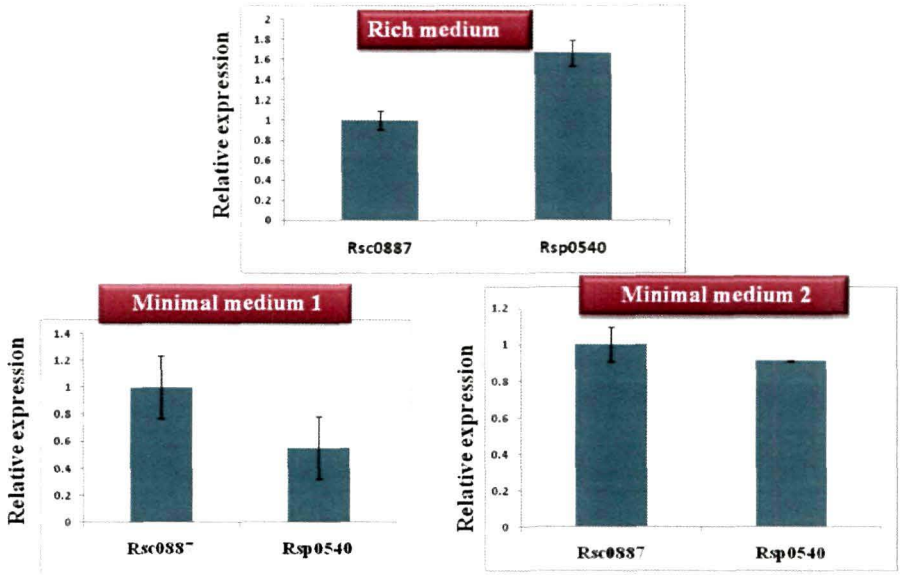


Fig. 4.9 Expression analysis of the gene RSc0887 and RSp0540 in Rich (BG) medium, minimal medium 1 and minimal medium 2. In rich media the expression of the RSp0540 is two fold more than RSc0887, In minimal media 1 result was observed just opposite to BG media. But in the minimal media the expression of both the gene are equal.

4.4.7 Change of surface morphology in RSc0887 and RSp0540 mutants

Filamentous hemagglutinins are known to remain adhered to the surface of a bacterium after secretion. Under scanning electron microscope photographs of BG grown F1C1 wild type strain, RSc0887 mutant (TRS1004) and RSp0540 mutant (TRS1008) were taken (Fig. 4.10). The surface structure of TRS1004 and TRS1008 were found to be rough in comparison to F1C1 wild type strain. From our expression data we had observed very low expression of these genes in BG medium. So the effect on the surface we observed in the mutants needs to be studied further either by complementation or by removal of the insertion or by creating more different mutations in these genes.

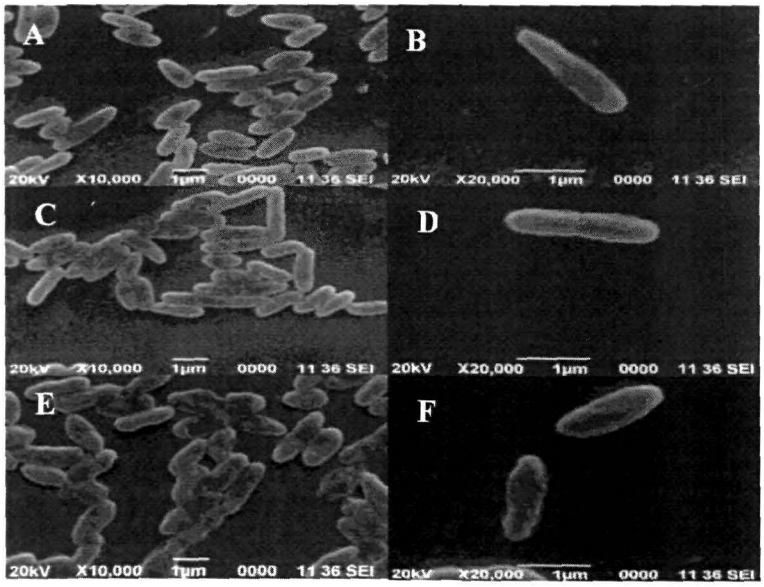


Fig. 4.10 Scanning electron micrograph of the F1C1, TRS004 and TRS008. Images were taken at different 10000 X and also 2000X. The SEM images shows that surface morphology of the mutants are found to be rough with respect to the control F1C1. (A) & (B) micrographs of control F1C1; (C) & (D) images showing TRS004 mutants surface morphology; (E) & (F) surface morphology of the TRS008

4.5 Discussion

In this study the occurrence of both the RSc0887 and RSp0540 in the F1C1 strain was found out. Independent insertion mutation in these two genes caused virulence deficiency. The virulence deficiency was due to mutation in these genes only or due to the polar effect of the insertion on the downstream genes cannot be told now. But there was difference between the two mutants with respect to their virulence deficiency. RSc0887 was more reduced than RSp0540 for virulence. Considering their high homology it can be assumed that the two proteins are secreted using RSc0888 encoded outer membrane protein. As RSc0888 is downstream to RSc0887, an insertion in RSc0887 is likely to affect RSc0888 expression. So insertion in RSc0887 might also affect RSp0540 expression by interfering with its transport across the outer membrane. This might be an explanation for the more virulence deficiency in case of RSc0887 than RSp0540. If our hypothesis is true then a double mutant having insertion in RSc0887 and RSp0540 will also exhibit virulence deficiency like RSc0887 mutant.

In spite of the presence of different genes encoding for hemagglutinin the genes RSc0887 and RSp0540 functions seems to be not redundant in F1C1. This might be the reason why one copy of the gene is conserved in all the *R. solanacearum* strains. Adhesion functions in bacterium may be involved in carrying out different functions such as attachment to the host cells, attachment to support while growing, attachment to other bacterial cells. Therefore, inspite of the presence of many adhesion functions, the role of specific adhesins may not be redundant. The mechanism of virulence deficiency and complementation studies with the above mutants will be done in near future. We will also doing mixed inoculation experiment with wild type F1C1 to find out whether the virulence deficiency is due to cell dependent or independent function. If any role of these genes different infecting different hosts will be done in near future by inoculating other host such as potato, egg plant and chili.

Expression studies of these two genes indicated that generally the genes are low expressed and they are not similar with their expression pattern in different media. As these encode for potential adhesion functions, expression of some adhesion function may be more in the presence of solid support than in liquid medium. *In planta* expression studies by *gus* gene fusion will be done in near future. In this regard the scanning electron micrograpgh indicating surface anomalies of both RSc0887 and Rsp0540 is intriguing, as these genes express very low under these conditions. It is known that bacteria deficient for non-fimbrial adhesion functions have surface anomalies[17]. As both these mutants are generated by insertion, in future complementation studies in the mutant will be to find out the contribution of these genes towards the surface phenotype.

4.6 References

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Chapter 5

Conclusion and Future Aspects

5.1 Conclusion

In this study, we have isolated and characterized a *Ralstonia solanacearum* strain. Standardization of techniques such as natural transformation, observation of twitching motility, cellulase activity, creation of insertion mutation, gene expression study by *lacZ* reporter gene fusion, seedling infection, GUS staining of bacteria *in planta* have been done to facilitate studying *R. solanacearum* virulence functions.

Our observation in this study regarding the possibility of the association of other endophytes with *R. solanacearum* in the wilted plant needs to be addressed carefully in near future. Our on-going research work suggests that some of the bacteria are able to colonize successfully into the tomato seedling (unpublished data). We are now trying to find the co-localization of *R. solanacearum* and the other endophyte (e.g. *Klebsiella*) in infected seedling. Virulence functions required to infect grown up tomato might not be always same as to infect seedlings. Therefore we are going to study virulence of *hrp* deficient mutant on tomato seedlings.

The hemmaglutinin mutants are found to be virulence deficient. Now we are subjecting its co-inoculation with wild type to know whether they can co-migrate or not. We are also trying to create double mutant of RSc0887 and RSp0540 and study the virulence phenotype of these mutants. It will be of our interest to find out the reason for their virulence deficiency. Adhesion functions are not only required for host cell attachment, but also for attachment between two bacterial cell, adhering to the bacterial biofilm.

The seedling assay that we have described in this study is likely to be useful to study gene expression *in planta*. The *R. solanacearum* RNA can be directly isolated from the wilted seedling in this method to study *in planta* gene expression.

In conclusion we would like to say that there are many aspects still need to be uncovered of this pathogen.

5.2 Future aspects

One of the important works that will be done in near future is getting the whole genome sequence of the *Ralstonia solanacearum* F1C1 strain. The genomic information will facilitate to carry out molecular genetic studies of the different genes and characterization of different hypothetical genes present in this bacterium. The genome information will be useful to do comparative study with the already sequenced genomes.

Gene expression inside the host plant will enable to understand pathogen adaptability to the host environment. It will also be helpful to understand *R. solanacearum* dynamics inside the host plant. Our interest is to study *in planta* gene expression in *R. solanacearum*. The seedling infection assay described in this study might be helpful to decipher if the bacterium has any preferred niche inside the plant.

The rate of movement of the bacterium inside the plant is not known. We will be trying to measure this rate by using the *gus* marked *R. solanacearum*.

R. solanacearum colonizes the entire seedling or plant upon infection. The seedling infection will help us in finding if this colonization process is uniform inside the host or there are some preferred niches for this bacterium. This will further lead us to know whether *R. solanacearum* gene expression is similar in different parts of the plant or not?

There are several unanswered questions that need to be answered in *R. solanacearum* research. We will be using the seedling infection as a model to address some of these questions in future.

APPENDIX I

1.Media Composition

(i) BG	Concentration(gl^{-1})
Peptone	10.0
Casamino acid	1.0
Yeast extract	1.0
Agar	15.0

25 ml of 20 % Glucose solution and 5 ml of TZC is added in 1 litre of BG media.

(ii) PSA	Concentration (gl^{-1})
Peptone	10.0
Sucrose	10.0
Agar	10.0

(iii) Minimal Media	Concentration (gl^{-1})
$FeSO_4\ 7H_2O$	1.25×10^{-4}
$(NH_4)_2SO_4\ 7H_2O$	0.5
$MgSO_4\ 7H_2O$	0.05
KH_2PO_4	3.4

pH 7 is adjusted with KOH.

(iv) LB	Concentration (gl^{-1})
Casein enzymic hydrolysate	10.0
Yeast extract	5.0
NaCl	5.0

(iv) Minimal medium 2 (SE medium)	Concentration
Soil	100 gm
Sterile water	200 ml

100 gm of garden soil was taken and 200 ml of sterile distilled water was added and mixed/vortexed properly. Then it was allowed to settle and filtered with filter paper. The filtrate was then diluted 10 fold with sterile water. The diluted mixture was then autoclaved and used as minimal medium 2 (SE medium)

2. Reagents, Buffers and Solutions

(A) Buffers

(i) 50X TAE electrophoresis buffer	Concentration (gl^{-1})
Tris base	242.0 gm
0.5 M EDTA	100.0 ml
Glacial acetic acid	57.1 ml
(ii) Phosphate buffer	Concentration (M)
$Na_2HPO_4\ 7H_2O$	0.06
$NaH_2PO_4\ H_2O$	0.04

pH was adjusted to 7.0 and was kept in RT.

(iii) Z- buffer	Concentration (M)
$Na_2HPO_4\ 7H_2O$	0.06
$NaH_2PO_4\ H_2O$	0.04
KCl	0.01
$MgSO_4$	0.001
B-mercaptoethanol	0.05

Final volume was made up to 50 ml and p^H was adjusted to 7.0. Buffer was stored in 4°C.

(iv) PBS buffer (1X)	Concentration (mg/ml)
NaCl	8.0
KCl	0.2
Na_2HPO_4	1.44
KH_2PO_4	0.24

(B) Reagents

(i) Ethidium bromide (stock)	Concentration (mg/ml)
Ethidium Bromide	10.0
(ii) IPTG	100.0
(iii) X- gal	50.0 (in DMF)
(iv) X- gluc	52.18(in DMF)
(v) ONPG	4

Dissolved in 0.1 M Phosphate buffer

(vi) Congo-red	Concentration (% w/v)
Congo-red	0.1

(vii) Antibiotics (Stock)	Concentration(mg/ml)
Ampicillin	50.0 (in water)
Gentamycin	50.0 (in water)
Rifampicin	50.0 (in DMF)
Spectinomycin	50.0 (in water)

(C) Solutions for plasmid DNA isolation

(i) Solution I	Concentration (gm %)
50 mM glucose	0.90
25 mM Tris-Cl (pH 8.0)	0.30
10 mM EDTA (pH 8.0)	0.37
(ii) Solution II	Concentration (gm %)
0.2 N NaOH	0.8
SDS	1.0
(iii) Solution III	Volume (ml)
5.0 M Potassium acetate	60.0
Glacial acetic acid	11.5
Autoclaved water	28.5

APPENDIX II

S.N	Name	Sequence	Specificity
1	Nmult:21:1F	CGTTGATGAGGCGCGCAATTT	Phylotype I (Asia)
2	Nmult:21:2F	AAGTTATGGACGGTGGAAGTC	Phylotype II (America)
3	Nmult:23:AF	ATTACSAGAGCAATCGAAAGATT	Phylotype III (Africa)
4	Nmult:22:InF	TTGCCAAGACGAGAGAAGTA	Phylotype IV (Tropical)
5	Nmult:22:RR	TCGCTTGACCCTATAACGAGTA	Reverse Primer
6	27F	GAGTTTGATCMTGGCTCAG	16S rDNA (F)
7	1525R	AAGGAGGTGATCCAGCC	16S rDNA (R)
8	oRK001	CGTGCTACAGGCGTCCACCG	RSc0887 (F)
9	oRK002	GAGCGGATTGGCGCTGGTGT	RSc0887 (R)
10	oRK007	ATGGACAGCGCGGCCTTGAC	RSp0540 (F)
11	oRK008	GGGCGGACACGGACAGGTTG	RSp0540 (R)
12	oRK009	CAGCGTCAACATCGGCGGGT	RSp0540 (F)
13	oRK010	TGCCGCTCGCATTGGTCTGG	RSp0540 (R)
14	oRK013	TCACGGATGGCGCGAAGCAG	RSp1071 (F)
15	oRK014	CGCCCGGCATCAAATGCATCC	RSp1071 (R)
16	oRK019	CGGTCAACAACAACAGCGCGTC	RSp1073 (F)
17	oRK020	CGTGCTGTCCTTGCGCCAGTT	RSp1073 (R)
18	oSKR33	TCAACAACAACTACAGCGCC	RSc0887 (qPCR)
19	oSKR34	GTACTGATAGAGCGTCCGCC	RSc0887 (qPCR)
20	oSKR37	ATACGCAGTTCAACGTTCCC	RSp0540 (qPCR)
21	oSKR38	TGACCTGGTTGACGATGATG	RSp0540 (qPCR)
22	oSKR45	CGTCAAGAACATGATCACCG	TuF (qPCR)
23	oSKR46	TGTCGCACTTGTTCAGGAAG	TuF (qPCR)

APPENDIX III

1. RSc0887 Sequence (1557 bp)

>Rsc0887

CGCAATGTCGGTGGCGGGCCGAACAGCAGCGGGGTGGGGCTTGCACCCTATGGCTCCGCTC
ACAGTGCGGACAACGCCGCCGGCAACAGCAGCCGCCAGAACGCGTCGGTCGTGATCGGCA
AGAGCGTGCAGGTGCAGGCGCGCACGGGCGACATCACCGTCTCGGGCAGTGGCATCTCGG
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TTACTCCGGCACGGTGGGCGTGCGCAGCGCCAGCAGCACGCTGGACACGGCCAAGAGCCA
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AGACGACGTAACGGTGCACGGTGCCGATGTGTCGGCCGGTGGGGACCTCAAGGTGACCGG
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GAGCCAGTATGGGGTGACGCTGGCGATGTCGGGCTATGCGGTGAGCATCGCGCAGTCGGT
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GGCGCAGGCGGCGTTGATGGGTTACAACGCTGCGGGGAATCCAGGTCTGAACTCCCAGAA
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CGGCAACGCGAACGTCGCGGAGGGCAAGACCGACAGCACGTATGCGTCGGTGGTGCATCA
GAGCGGTATCGCGGCGGGCACGGGCGGCTACAACATTAACGTGAAGGGCAACACGGATCT
GGTGGGCGGGGTGATCTCGTCCACGGCGGCGCCGAGCAAGAACGTGCTG

[Sequence yet to be submitted to NCBI]

2. RSp0540 Sequence (1566 bp)

>Rsc0540

CTTGCGCGCCTATCGCGAGCGCGGCGGGATTGCCGTGGTGACGACGGATTCCACGCCGTCC
GCCGATGAACTGGTGCGCGCATCGAGCGTTGAGCTTGCGCGGAGCGTGCATCTCACGGAG
GTGATGCCGCCGATCGGCAAGCGCAATGTGCGCCTGGTGCGGGCGTTCGTGGCCGACATGA
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GCACGGCGCTGCTGCAGCGCGGCCCGTTTGACCTGGGGGATTTTGAGATCCGATTCGGGCC
CGCTCAGTACGAGGGCTCGCGATACGTGGGCATCGGCATCATCGGCCACCTGGGACGGGTG
TTGAACTGACGATAGGGGGAGCACATTGGGCTTGCGTCGATGAGGTTTGTTGAGGGTTTGG
CGCAAGCTACGGCGGCTTCCGGCAATTTCGCTGTCTGGCTACCTCAAATTGAGTAGTTTTCTT
CACTTTGTGCAGTAAAGTGCGCTTAGAATCCCGGTCTTGGTAGAAATTACAAAGTCTGGGTT
GTCATGAACGCGAAGTGCTATCGAACCGTTTTCAACGCTGTACGCGGCATGTTGGTGGCCGT
AGAGGAATCGGCGAGAAGCACCGGCAAGGGGCGTCAGACCGGCGGGCAAGCCGGCACGT
CGGCCGCATCGACCTCCACCGCCGCGCGCTTCGCCGTGCTGCCGGTGGTGTTCGGGGCATG
GTGCGTGTTGGGCTTGCCCTACACGTGCAGGCCCAGGTCGTCGCGGCCCGGGGTCGGCGCC
CATGTCATCCAGACGCAAACGAATTGCAGCAAGTCAACATCGCCACGTCCAACGGAGCGGT
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TTCCCTGCGCGGTGTTGCCGCTGCCCAGGCGGGCGATCTGACGCT

[Sequence yet to be submitted to NCBI]

3. *gspD* Sequence (395 bp)

>gspD

AGCCGTAGGTGTTCGGTCCGCCCGCGCCCACCATCCACTGGATGCCCAGCTGCGACGCCTTG
TCGGAGGTCACCTCGACGATCATCGATTCGATATAGACCTGCGCGCGGCGCGCGTCGAGGT
CGTCGATCACCGTGCGCAGATTGCGGTACACCGGTTCGCTGGCCGTGATGATCAGCGCATT
GGTGGCCGGGTCGGCCTGGATGATGCCGCCGGTGGTCGGCAGGTTGCTCTGGCCGAACGA
GGCGCGGAACGACGAGTTGCCGCTGCCCATGCCACTGCTGCTGCCCGAGCTGCTGCCGTAG
CTGCCGGTCTGCGTGTTCTGGCTGGTCTGCGTGCCGGTGGTGGCAGGCTGCTGCGCCTGCG
CGCCCTGCGCCGCGCTCTGGCCGCCCGGG

[Sequence yet to be submitted to NCBI]

List of Publications

- **Kumar, R.**, Barman, A., Jha, G. & Ray, S. K. Identification and establishment of genomic identity of *Ralstonia solanacearum* isolated from a wilted chilli plant at Tezpur, North East India. *Curr. Sci.* **105**, 1571-1578, 2013.
- Ray, S. K., **Kumar, R.**, Peeters, N., Boucher, C. & Genin, S. *rpoN1*, but not *rpoN2*, is required for twitching motility, natural competence, growth on nitrate and virulence of *Ralstonia solanacearum.* (Communicated).

Other Publications

- Barman, A., **Kumar, R.**, Kumar, P. & Ray, S. K. Exclusion of replicative transposons from linear chromosome: an interesting observation. *Ind. J. Fund. Appl Life Sci.* 85-90, 2013.
- Chaturvedi, M., Singh, M., **Kumar, R.** & Chugh, M. R. Isolation of lipase producing bacteria from oil contaminated soil for the production of lipase bysolid state fermentation using coconut oil cake. *Int. J. Biochem. Biotechnol.* **6,** 585–595 , 2010.
- Chaturvedi, M., Singh, M., **Kumar, R.** & Chugh M. R. Seaweeds: A diet with nutritional, medicinal and industrial value, *J. Med. Plants Res.* 1819-3455, 2011.
- Phukan, M. M., Chutia, R. S., **Kumar, R.**, Kalita, D., Konwar, B. K. & Kataki, R. Assessment of antimicrobial activity of bio-oil from *Pongamia glabra*, *Mesua ferrea* and *Parachlorella* spp deoiled cake, *Int. J. Pharm. Biol. Sci.* **4** ,910, 2013.

Identification and establishment of genomic identity of *Ralstonia solanacearum* isolated from a wilted chilli plant at Tezpur, North East India

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The bacterial wilt disease caused by *Ralstonia solanacearum* is an ever-increasing threat to tropical as well as temperate regions of the world. Though the disease has been reported from different parts of India, appropriate identification of the pathogen at molecular level is still incomplete. In this study we report the isolation and molecular characterization of a *R. solanacearum* strain F1C1 from wilted chilli plant collected from a field near Tezpur University, Assam, India, using techniques such as multiplex PCR, 16SrDNA sequencing, multilocus typing, pathogenicity test, twitching motility and natural transformation. Our results suggest that F1C1 is a phylotype-I strain of *R. solanacearum* species complex. Additionally, we also report presence of other bacterial species in the ooze collected from wilted plants.

Keywords: Bacterial wilt, genomic identity, plant pathogenic bacteria, *Ralstonia solanacearum*.

RALSTONIA SOLANACEARUM is a destructive bacterial phytopathogen belonging to the class β-Proteobacteria. It causes wilt disease in more than 450 plant species of 54 botanical families across the globe[1]. Owing to its wide host-range, long persistence in soil, extensive geographical distribution and profuse pathogenic nature leading to severe loss of various economically important crops, *R. solanacearum* has been ranked second among the top-ten devastating plant-pathogenic bacteria[2,3]. The pathogen is evolving faster and a large number of new strains have been reported quite regularly. Considering the genetic diversity among the strains responsible for the wilting disease in different plants, the pathogen is now termed as *R. solanacearum* species complex[4]. In a traditional way this pathogen has been classified into five races with respect to their host specificity[5–7] and six biovars according to their biochemical properties[6,8,9]. RFLP map[10,11] has been utilized to further divide the species complex into 'Americanum' (containing biovars 1, 2 and N2 strains) and 'Asiaticum' (containing biovars 3, 4 and 5 strains) divisions respectively. Lately, the bacterium has been categorized into four phylotypes and 23 sequevers based on phylogenetic analysis of 16S–23S internal transcribed spacer (ITS) region, but there is lack of a general agreement on sub-classification of the pathogen[12].

Since the initiation of *R. solanacearum* research in the early fifties[13], several aspects relating to the pathobiology of this bacterium have been enlightened[2,4,14–19]. The first strain of this pathogen to be sequenced in 2002 was a race 1 isolate from tomato plant[20], called GMI1000. Till date four strains of *R. solanacearum* have been sequenced with chromosome and plasmid annotation completed and another six strains with contig sequences (NCBI; http://www.ncbi.nlm.nih.gov/genome/genomes/490). No genome sequences of the pathogen from the Indian subcontinent are available yet. Published literature on prevalence of *R. solanacearum* species complex from India is scarce and ample exploration of this important phytopathogen is still lacking. There is not a single strain of *R. solanacearum* available at the Microbial Type Culture Collection and Gene Bank (MTCC) in IMTECH, Chandigarh (http://mtcc.imtech.res.in/catalogue.php), which is the national repository of microbes in India. Chattopadhyay and Mukhopadhyay[21] reported bacterial wilt of banana (Moko disease) in West Bengal. Since then no seminal work on the pathogen can be traced from this subcontinent, although economic losses due this pathogen are immense. Reports on taxonomic classification of this bacterium from India is not many. Grover *et al.*[22] have utilized short tandem repeats (STRs) at specific loci as markers to identify *R. solanacearum* isolates. Recently, Chandrashekara *et al.*[23] have differentiated 57 isolates of *R. solanacearum* from different wilted host plants into a race on the basis of their pathogenicity, 16SrDNA sequence and serological tests. Kumar *et al.*[24] have performed molecular analysis of 33 strains of *R. solanacearum* obtained from Karnataka, Kerala, West Bengal and Assam by REP–PCR, ITS–PCR and RFLP–PCR dividing them into various clusters.

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It is important to note that use of different genotype and phenotype approaches is important for correct identification of bacterium at the species level as 16SrDNA sequencing is not always the best approach for correct identification of bacterial isolates at species level[25–27]. Therefore, in this work, apart from the 16SrDNA sequencing, we have utilized the widely accepted molecular method of multiplex-PCR with phylotype specific primers[12] and multilocus typing to identify *R solanacearum* from wilted host-plants.

Materials and methods

Collection of wilted plants

The wilted plants were collected from the chilli-grown fields near Tezpur University campus, Assam, India (26.63°N 92.8°E). The plants were collected after critically observing typical wilting symptoms. More than ten fields were surveyed and wilted brinjal plants (egg plant), chilli plants, potato plants and tomato plants were collected (Figure 1).

Isolation of bacteria

Wilted plants collected were washed with clean tap water to remove surface soil. Approximately 10 cm stem was cut from the plant and rinsed with distilled water thrice, following which it was surface sterilized with 70% ethanol. The ethanol-swabbed stem portion was then rinsed with sterile water to remove ethanol from its surface. The stem was cut in the middle using sterile scalpel and one of the cut ends was dipped into sterile water in a test tube. After 10–15 min time interval, streams of white-coloured ooze could be seen coming out of cut end. The ooze was then collected, serially diluted 10^6 fold and plated on the peptone sucrose agar (PSA) plate containing 2,3,5-Triphenyl Tetrazolium Chloride (TZC). All the plates were incubated at 28°C for 48 h and observed for the appearance of reddish/pinkish centred mucoid colonies.

Bacterial growth media

PS (1% peptone, 1% sucrose, 1.6% agar in solid medium; percentage in weight per volume) medium was used for the culturing the bacterial isolates from wilted plants. Later, standardized Phi (1% peptone, 0.1% yeast extract, 0.1% casamino acid, 1.6% agar in solid medium; percentage in weight per volume) medium was used for culture of *R. solanacearum*[28]. To 200 ml Phi medium, 1 ml of 1% TZC (autoclaved separately) and 5 ml of 20% glucose (autoclaved separately) were added for observing *R. solanacearum* pinkish/reddish centred colony morphology. All the chemicals and growth media components were obtained from HiMedia (Mumbai, India), except casamino acid (SRL, Mumbai, India). For selection of *R. solanacearum* transformants, 50 μg/ml spectinomycin (HiMedia, Mumbai, India) concentration was used in the media.

Twitching motility study

For observing twitching motility, F1C1 was streaked in quadrants to get a decreased concentration of the bacterium on solid Phi medium. After overnight incubation (18–24 h), the plates were observed under the compound microscope with 4X objective. At the edges of the bacterial streaking finger-like projection of bacterial growth which is a surface translocation of cells was observed. The twitching motility ceases in older colonies.

Pathogenicity assay on tomato plant

The bacterial isolate was checked for degree of infectivity on tomato plants (PUSA RUBY variety) grown in earthen pots. The plantlets were one month old after seedlings were planted separately. For inoculation, bacteria were grown in PS medium at 28°C for 48 h. Then 1 ml of this culture was pelleted down; the pellet was suspended in 1 ml sterile water and mixed by gentle pipetting. A sterile syringe needle was dipped into this culture and was used to prick the stem of tomato plants, just above the cotyledon leaves. A set of 20 plants was taken as negative control and 40 plants were taken for bacterial inoculation. Control plants were inoculated by stem pricking with sterile needle dipped in sterile water. Wilting score was done from the day the first wilting symptom was noticed.

Polymerase chain reaction

Phylotype specific multiplex PCR: As described by Fegan and Prior[12], multiplex PCR was performed using five different phylotype specific primers:

(i) Nmult: 21 : 1F: CGTTGATGAGGCGCGCAATTT,
(ii) Nmult: 21 : 2F: AAGTTATGGACGGTGGAAGTC,
(iii) Nmult: 23 : AF: ATTACSAGAGCAATCGAAAGATT,
(iv) Nmult: 22 : InF: TTGCCAAGACGAGAGAAGTA,
(v) Nmult: 22 : RR: TCGCTTGACCCTATAACGAGTA.

Each PCR reaction was set in 15 μl reaction volume consisting of 1.5 μl of 10× *Taq* buffer (1.5 μl of 15 mM $MgCl_2$ was added separately to the reaction mixture), 1.5 μl of 2 mM dNTP mix, 0.2 μl of *Taq* polymerase (5 U/μl), 1 μl of 10 μM primer (Sigma Aldrich, India) and finally the volume was adjusted to 15 μl with sterile de-ionized water. To the above reaction mixture, 1 μl of bacterial

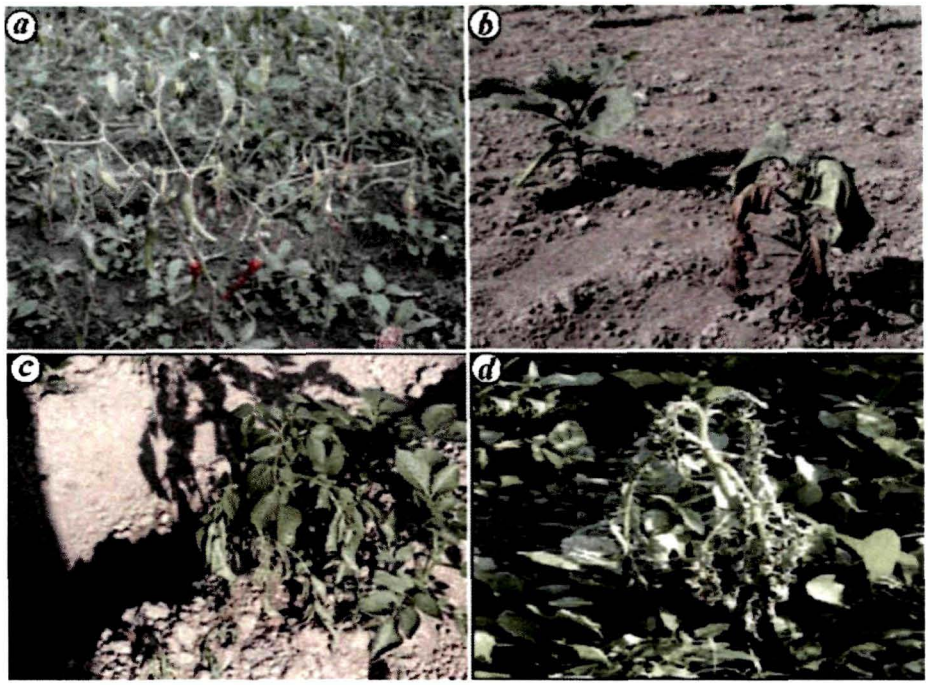

Figure 1. Wilted plants from different crop fields. ***a***, Wilted chilli plant. ***b***, Wilted potato plants along with some healthy potato plants. ***c***, Wilted brinjal plant (egg plant) along with a healthy brinjal plant in the same field. ***d***, Wilted tomato plant.

suspension was added as template (bacterial suspension was obtained by suspending single bacterial colony in 95 µl water followed by addition of 5 µl of 200 mM NaOH and incubation at 95°C for 10 min). PCR parameters for DNA amplification comprised overall 35 cycles: initial heating at 96°C for 5 min, denaturation at 94°C for 15 sec, annealing at 59°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for 10 min in a thermal cycler (Applied Biosystems; Veriti, USA). The amplified product was analysed in 2% agarose gel and was documented (Gel doc, UVP, USA).

16SrDNA amplification: Amplification of 16SrDNA gene was performed using 16SrDNA specific primers: 27f (5′-GAGTTTGATCMTGGCTCAG-3′) and 1525r (5′-AAGGAGGTGATCCAGCC-3′)[29]. PCR conditions used were – step 1: 96°C – 2 min, step 2: comprising 34 cycles of 94°C – 30 sec, 55°C – 1 min, 72°C – 1 min, step 3: 72°C – 10 min (laboratory of Dr S. Genin, France). The amplified DNA product was purified using quick-spin PCR purification kit (Qiagen, Tokyo, Japan). The purified product was then sequenced using the sequencing facility (Applied Biosystems) at Tezpur University. The sequence was submitted to GenBank.

Multilocus typing: Gene-specific primers corresponding to loci RSc0887, RSp0540, RSp1071 and RSp1073 of *R. solanacearum* GMI1000 strain were designed to check for amplification in the specific gene sequences in F1C1.

RSc0887: CGTGCTACAGGCGTCCACCG (oRK001) and GAGCGGATTGGCGCTGGTGT (oRK002);

RSp0540: ATGGACAGCGCGGCCTTGAC (oRK007) and GGGCGGACACGGACAGGTTG (oRK008); CAGCGTCAACATCGGCGGGT (oRK009), TGCCGCTCGCATTGGTCTGG (oRK010), no amplification occur using this pair (oRK009 and oRK010) of primers (Figure 2);

RSp1071: TCACGGATGGCGCGAAGCAG (oRK013) and CGCCCGGCATCAAATGCATCC (oRK014);

RSp1073: CGGTCAACAACAACAGCGCGTC(oRK019) and CGTGCTGTCCTTGCGCCAGTT (oRK020).

Sequences were retrieved from https://iant.toulouse.inra.fr/bacteria/annotation/cgi/ralso.cgi. PCR amplification consisted of total 35 cycles: initial heating at 95°C – 5 min, denaturation at 94°C – 1 min, annealing at 58°C – 30 sec and extension at 72°C – 2 sec and final extension at 72°C – 10 min in a thermal cycler (Applied Biosystems, Veriti, USA).

Natural transformation

F1C1 competent cells were prepared as described by Plener *et al.*[28]. FIC1 was inoculated in Phi medium and allowed to grow for 48 h. Then 100 µl from the grown culture was added in 10 ml minimal medium (g l^{-1}: $FeSO_4$–$7H_2O$, 1.25×10^{-4}; $(NH_4)_2SO_4$, 0.5; $MgSO_4$ – $7H_2O$, 0.05; KH_2PO_4, 3.4; pH adjusted to 7 with KOH) containing 600 µl of 60% glycerol. As the cells grow slowly in minimal medium, turbidity of the medium does not change significantly. After 48 h of growth, 100 µl of the culture was added with 5 µg of the plasmid pRK1001

(unpublished result; with spectinomycin resistance gene). The mixture was put on top of a nylon membrane placed over solid Phi medium. The cell suspension was allowed to dry inside the flow bench. The plate was incubated for 48 h at 28°C. The grown cells from the nylon membrane were mixed in 100 µl of sterile water, which was later plated on solid Phi medium containing TZC, glucose and spectinomycin.

Results

The ooze collected from wilted plant is a mixture of different bacteria

A typical test for bacterial wilt is the observation of whitish ooze streaming out from the cut end of the infected stem after 15–20 min of exposure to water. As expected the wilted plants collected from the fields tested positive for bacterial wilt. To find out bacterial presence, the whitish ooze streaming out of the cut end of the wilted plant stem was collected in a test tube. The ooze was then serially diluted maximum to 10^6 fold and then plated on TZC + PSA plate. Bacterial colonies were observed to appear at different intervals of incubation time such as 24, 48 and 72 h. Some colonies that appeared on the plate were white, dark pink and others included white with pinkish centred colonies. *R. solanacearum* is known to form pink centred mucoid colony. All the bacteria with mucoid and pink centred colonies that appeared after 24, 48 and 72 h after plating were preserved. Interestingly, colonies that appeared after 24 h and 48 h had similar morphology. The bacterial colonies with pink centre and mucoid nature were further streaked on the plates to get a pure colony and then stored for further studies. It was clear from the colony morphology and growth appearance of the colonies that the ooze contains different kinds of bacteria. We collected a total of 400 bacterial isolates from different wilted plants.

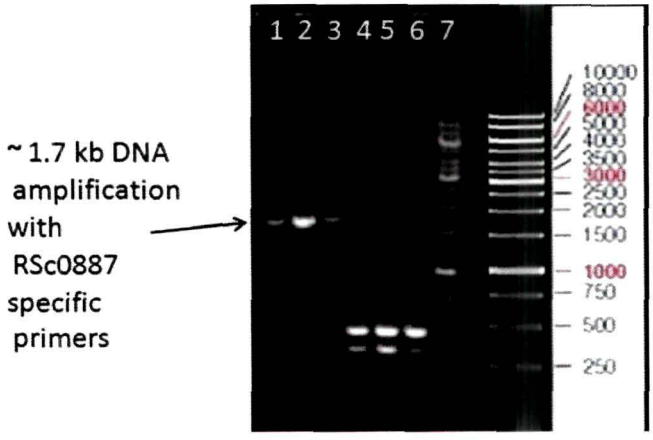

Figure 2. Gel photographs demonstrating amplification of a desired size DNA in FICI genome using oligo designed against RSc0887 locus of GMI1000 genome. Lane 7, 1 kb gene ruler (Fermentas, UK). The size of different DNA markers is given by the side. Lanes 1–3, Amplification of ~1.7 kb DNA from RSc0887 homologue in F1C1 (oligos used were oRK001 and oRK002). Lanes 4–6, No amplification of expected size DNA fragment using oligos oRK009 and oRK010 designed against RSp0540 locus of GMI1000. No amplification in lanes 4–6 may be due to sequence difference between GMI1000 and F1C1 genomes at the primer binding region. However, RSp0540 locus in FICI has been confirmed by amplification results obtained with another set of oligos (oRK007 and oRK008; data not shown).

Molecular identification of R. solanacearum among the bacterial isolates

To identify the *R. solanacearum* among the bacterial isolates we utilized the widely accepted method of multiplex PCR using phylotype-specific primers[12]. In this method, a *R. solanacearum* strain belonging to any of the four phylotypes can be identified by observing the amplification of the different sized phylotype-specific DNA fragments. This method has been used in the molecular identification of many *R. solanacearum* isolates[12]. Out of total 400 isolates taken for the multiplex PCR analysis, amplification of DNA band was observed only in four isolates. All the four isolates yielded ~144 bp size DNA fragment that resembled the standard amplification product reported from *R. solanacearum* belonging to phylotype-I (Figure 3). One of the isolates is from a wilted chilli plant, which we refer to as F1C1 (F1: field surveyed 1, C1: colony no. 1 isolated from chilli plant), the second is from a wilted tomato plant which we refer to as F3T23 (F3: field surveyed 3, T23: colony no. 23 isolated from tomato), the third and the fourth ones are from a wilted potato plant, collected from Jagatsinghpur district, Odisha, India. To confirm the phylotype-specific DNA amplification, the experiment was repeated three times with each the four strains. This result of the multiplex PCR is in agreement with the conclusion of Fegan and Prior[12], that

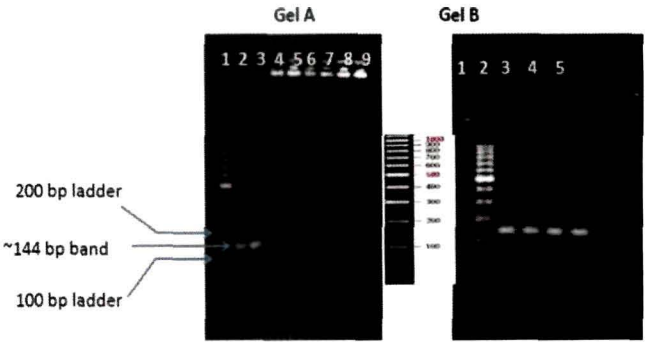

Figure 3. Gel photograph of multiplex PCR of various bacterial isolates. ***a***, Lane 1, Gene ruler (Fermentas, UK). The size of the markers from the lower to upper end of the gel is 100, 200, 300 bp, etc. Lanes 2, 3, Amplification of the phylotype-I-specific ~144 bp in F1C1. Lanes 4–9, No amplification from other bacterial isolates collected from wilted plants. ***b***, Lane 1, 100 bp gene ruler (Fermentas, UK). The size of the markers from the lower to upper end of the gel is 100, 200, 300 bp, etc. Lanes 2–5, Confirmation of phylotype-I-specific band amplification in four bacterial isolates, including F1C1 in lane 2.

phylotype-I *R. solanacearum* strains are of Asiatic origin. All the four above isolates, exhibiting the amplification of the phylotype-I-specific band in multiplex PCR were found to grow slowly on PSA plates. After streaking on PSA plates, single colony appeared only after 48 h of incubation at 28°C. This is in confirmation with the slow growth rate of *R. solanacearum*.

Out of the four bacterial isolates, F1C1 was taken for further characterization. We amplified the 16SrDNA from F1C1 using universal primers. Partial sequence obtained from the amplified product exhibited 100% homology to 16SrDNA of different *R. solanacearum* strains. The sequences were submitted to GenBank and the accession numbers are BankIt1610759 Seq1 KC755042 and BankIt1610759 Seq2 KC755043.

To further confirm F1C1 as *R. solanacearum*, we tried to partially amplify some of the potential pathogenicity genes in this bacterium. Primers were designed against the four hemagglutinin genes *RSc0887*, *RSp0540*, *RSp1071* and *RSp1073* of the GMI1000 genome. The GMI1000 genome was followed for designing primers because it belongs to phylotype-I of *R. solanacearum* species complex and it was evident from multiplex PCR that F1C1 also belongs to the same group. After PCR with primers specific to different loci, amplified product of the desired size was observed in the gel. Figure 2 (lanes 1–3) depicts the amplification of the expected 1.7 kb size DNA band from RSc0887 homolog from F1C1 genome. In Figure 2 (lanes 4–6) amplification of DNA band with expected size could not be observed. On this occasion the oligos designed against RSp0540 locus of GMI1000 might have failed to pair completely with the genomic locus in F1C1 isolate. The possibility of absence of RSp0540 in F1C1 was eliminated since amplification of the expected sized DNA fragment (1.7 kb) with another pair of oligos designed against RSp0540 of GMI1000 was achieved. We also got partial sequence of the two amplified products. As expected, the sequence exhibited very high homology at nucleotide level with RSc0887 and RSp0540 loci in *R. solanacearum* genome sequence.

Apart from RSc0887 and RSp0540, amplification of expected sized DNA bands, i.e. 1.8 kb were also observed for RSp1071 and RSp1073 homologs in F1C1. We tried to amplify long-sized DNA regions (1.7 kb or more) from F1C1 genome considering its future use in homologous recombination for gene insertion mutation. The experiment was repeated several times to confirm the amplification result. Although the amplified DNA regions expected from RSp1071 and RSp1073 homologs are yet to be sequenced, the size of the amplified DNA strongly indicates the presence of the above GMI1000 homologs in F1C1. More regions from the F1C1 genome have now been amplified in our laboratory using oligos designed against the GMI1000 genome, which further indicates the presence of the homologous loci in the F1C1 genome.

Twitching motility, transformation and pathogenicity test of F1C1

R. solanacearum has been reported to exhibit twitching motility[30]. So, we also looked for twitching motility in FICI. F1C1-streaked plates were observed after 24 h of incubation, under a compound microscope with an 4X objective. Finger-like projections (Figure 4 *b*) emerging out of the streaked edges were observed on the plates, suggesting F1C1 is capable of manifesting twitching motility. Twitching motility is basically due to the presence of type-IV pili on Gram-negative bacterial cell envelope[31], and *R. solanacearum* demonstrates identical features. As a control the common laboratory strain *Escherichia coli* DH5α, a *Lysinibacillus* species (isolated from wilted plant; this study) was observed to be negative for twitching motility.

R. solanacearum develops natural competence for taking external DNA molecules. Therefore, it is easy to knock down genes in this bacterium by homologous recombination. Twitching motility is important for natural transformation in this bacterium because mutants deficient for twitching motility are transformation-inefficient. As F1C1 is proficient for twitching motility, we studied natural transformation in this bacterium. We used a plasmid pRK1001 (unpublished result) to transform F1C1. The plasmid carries a partial *RSc0887* gene sequence within which an omega cassette (resistant for spectinomycin) has been inserted. The linearized pRK1001 was used to naturally transform F1C1. Transformants were selected on Phi containing spectinomycin. Totally 120 spectinomycin-resistant colonies were found in bacteria where the plasmid was added, whereas in the control in which no plasmid was added to competent F1C1 cells, not a single spectinomycin-resistant colony was found. Transformation experiment was also done with other plasmid constructs (unpublished result) and the result suggested that F1C1 is efficient for natural transformation like other *R. solanacearum* strains[32].

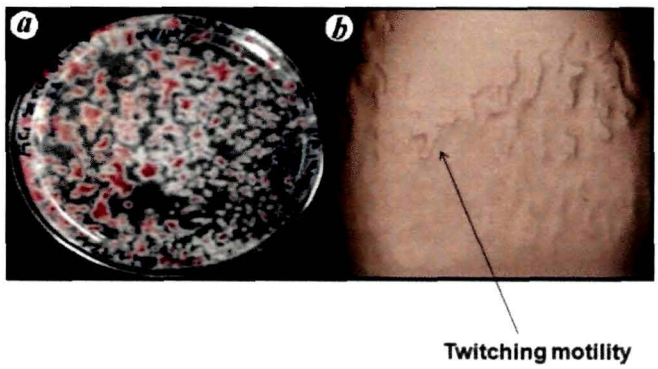

Figure 4. FIC1 growth on Phi plate. (***a***) On TZC-containing Phi plate F1C1 forms pinkish centred colony having white periphery. (***b***) Twitching motility in F1C1. Finger-like projections (called twitching motility) from the edges of the bacterial growth are observed after 24 h streaking on Phi medium. Using a compound microscope with 4X objective.

R. solanacearum is known for its broad host range ability in causing wilting disease. F1C1 is an isolate from chilli. We tested its pathogenicity on tomato plants. Control and F1C1-inoculated plants were observed the next day onwards following inoculation. Wilting symptoms were given numerical values 0 to 4 according to the degree of disease phenotype observed: 0 indicates no wilting and 4 indicates complete wilting of the plant. The wilting scores are given in Figure 5. On the seventh day post-inoculation, complete wilting symptoms were visible in several inoculated plants and tomato plants were seen to be dying (Figure 6). In case of inoculated plants, around 25% plants died due to wilting, some wilted partially and some had no symptoms of wilting. In the case of control, none of the plants exhibited wilting symptoms. The plants that wilted after inoculation with F1C1 were collected and streaming of the whitish ooze was observed which confirmed that the wilting was due to bacterial infection.

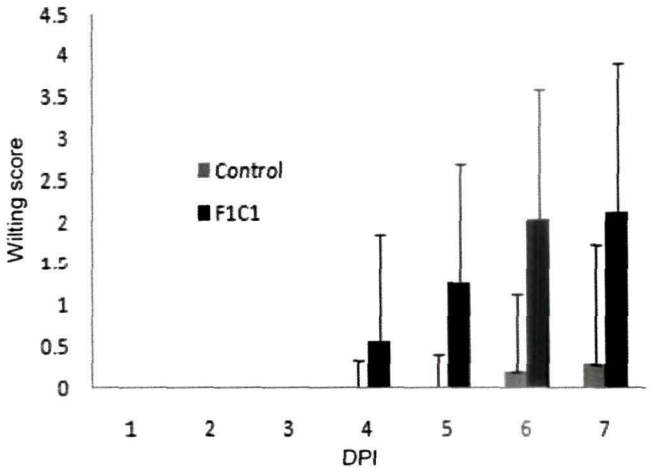

Figure 5. Wilting score till seven days post-inoculation. Totally 40 plants were inoculated with F1C1 by stem pricking and 20 plants were inoculated with sterile water by stem pricking. Wilting was scored using a scale 0.0 (for no or 0% wilting) to 4.0 (for 100% wilting). First wilting symptoms appeared after four days post-inoculation (DPI). After seven DPI while the wilting score in the F1C1 plants was 2.5, for the water-inoculated plants it was only 0.15. Error bars shown are the standard deviation values.

Figure 6. Photographs of F1C1 inoculated wilted tomato plants. A wilted tomato plant (right) after seven days post-inoculation with F1C1. A healthy tomato plant (left) after seven days post-inoculation with sterile water. The F1C1-inoculated wilted plant was positive in the ooze test.

Presence of other bacteria in the ooze

From multiplex PCR analysis many of the bacteria were found to be different from *R. solanacearum*. We amplified 16SrDNA in 15 different bacterial isolates that were similar to *R. solanacearum* with respect to colony morphology. All the bacteria were identified as *Lysinibacillus* (unpublished result). Two of the isolates were observed to promote plant growth upon inoculation (unpublished result). The other 13 bacteria are yet to be tested on plants. In a separate experiment, we observed a slow-growing bacterium was inhibiting the growth of a fungus as a contaminant on the plate. We confirmed its antifungal activity against few fungal pathogens (unpublished result). After 16SrDNA analysis, the bacterium was identified as *Alcaligenes* species. There are many other bacteria yet to be identified. The presence of other bacteria in the ooze along with *R. solanacearum* is intriguing. Though we had collected the ooze from the wilted plant after surface sterilization, the endophytic origin of the other bacteria that were isolated along with *R. solanacearum* in the ooze cannot be claimed with certainty. The possibility that these bacteria were localized on the surface of the wilted plant and had escaped the surface sterilization cannot be ruled out. In future independent inoculation experiments of these bacteria in plants as well as inoculation of these bacteria along with *R. solanacearum* followed by localization study in plants will prove their association with *R. solanacearum* during infection.

Discussion

In this work, we identified a *R. solanacearum* strain from wilted chilli plant. Apart from characteristic phenotypic studies such as growth, colony phenotype on TZC medium, twitching motility and pathogenicity test on tomato plant, we used molecular techniques such as 16SrDNA sequencing, phylotype-specific primer-aided multiplex PCR and multi-loci typing to confirm the strain as a member of *R. solanacearum* F1C1 belongs to phylotype-I of the *R. solanacearum* species complex. This finding is in agreement with the geographical distribution of the pathogen according to which phylotype I is known to be of Asiatic origin[12].

In the pathogenicity experiment we did not observe 100% wilting in all the infected plants. This is a usual observation in *R. solanacearum* infection study (S.K.R. had personal experience while working with *R. solanacearum* GMI1000 at LIPM, CNRS-INRA, France). Why some plants escape wilting symptoms (escapees) is not

known? Whether the bacterium survives inside these escapees has not been investigated. Recently, it has been reported that *R. solanacearum* can grow inside resistant *Arabidopsis thaliana* without causing wilting[33]. But finding the bacterium inside a susceptible host and not causing disease will be an interesting future aspect of our research.

At present complete genome sequences of only four strains of *R. solanacearum* are available in the public database. Except GMI1000, which is an isolate from French Guyana (South America), the other three strains do not belong to phylotype-I. Significant diversity exists among different phylotypes[34]. The whole genome sequence of F1C1 and studying its relative diversity with other sequenced strains will be interesting from the view of understanding its evolution and origin. This is also expected to illuminate different facets of the bacterium such as intricate virulence functions, adaptive mechanisms for persistence in this particular geographical location, phylogenetic relationships with already evolved and evolving strains, etc.

One of the important aspects we have observed during this isolation process which has been ignored or omitted in previous literature is the description of persistence of several other bacteria in ooze emerging out of the cut end of the wilted stem. In fact, the population of *R solanacearum* was found to be very low in the ooze collected, as only four positive isolates were found from the 400 isolates stored. There is no report available in the literature regarding the quality and quantity of other bacterial association during *R. solanacearum* infection. The slow growth rate of the bacterium may be a reason for our failure to obtain more of it from the infected plant. A Gram-positive bacterium of *Lysinibacillus* species was observed to be the predominant ingredient of the ooze. This bacterium appears after overnight incubation in rich medium but forms the characteristic pink centred colonies on TZC plate, which resembles that of *R. solanacearum*. As *Lysinibacillus* grows faster and the colony is mucoid in nature, this bacterium covers the whole plate and makes it difficult to identify *R. solanacearum* in the plate. Growth rate observation is critical to differentiate both the bacteria. In addition, the other simple approach might be used (which we did not try in this study), i.e. diluting the collected ooze to 10^7-, 10^8-fold before plating. This might reduce the load of other bacteria leaving only the most abundant bacterium, which is likely *R. solanacearum*. As evident in the literature, we also observed the *Lysinibacillus* bacterium isolated during this study to promote plant growth upon soil inoculation as well as stem inoculation (unpublished data). Another constituent bacterium belonging to *Alcaligenes* species exhibited significant antifungal activity against few destructive fungal phytopathogens (unpublished result). Whether these bacterial species isolated from the wilted plants remain associated with *R solanacearum*, is not known. We also do not know their exact localization in the plants. The possibility that these bacteria are surface-localized and have escaped the surface sterilization during ooze-collection process cannot be ignored. However, isolation of *Lysinibacillus* species from different wilted plants, observation of its plant growth promotion activity upon independent inoculation in plants (unpublished data), and information from the literature regarding its plant promoting activity, indicate its endophytic origin.

The environment inside plant xylem is considered as nutritionally poor and oxygen-limiting[35]. Therefore, microorganisms such as *R. solanacearum* that have evolved adaptive features to survive under these circumstances were expected to out-compete other bacteria here. From the recent studies, it is clear that inside the plant xylem *R. solanacearum* is in constant crosstalk with plant cells[36]. A recent study on *R. solanacearum* gene expression indicates the availability of sucrose for the bacterium inside the plant xylem[36]. So the abiotic and biotic environment of xylem after and before invasion of *R solanacearum* is going to be an interesting aspect of future research.

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