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\textsuperscript{5, 6, 7} and six biovars according to their bio-chemical properties\textsuperscript{6, 8, 9}. RFLP map\textsuperscript{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\textsuperscript{12}.

Since the initiation of \textit{R. solanacearum} research in early fifties\textsuperscript{13} several aspects relating to the pathobiology of this bacterium have been enlightened\textsuperscript{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\textsuperscript{20}, called GMI1000, in 2002. Till date four strains of \textit{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\textsuperscript{21}. Published literature on prevalence of \textit{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 \textit{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\textsuperscript{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 \textit{et al.} (2011) have utilized short tandem repeats (STRs) at specific loci as markers to identify \textit{R. solanacearum} isolates\textsuperscript{23}. Recently, Chandrashekara \textit{et al.} (2012) differentiated fifty-seven isolates of \textit{R. solanacearum} from different wilted host plants into a
race on the basis of their pathogenicity, 16S rDNA sequence and serological tests\textsuperscript{24}. Kumar \textit{et al.} (2004) have performed molecular analysis of 33 strains of \textit{R. solanacearum} obtained from Karnataka, Kerala, West Bengal and Assam by REP-PCR, ITS-PCR and RFLP-PCR dividing them into various clusters\textsuperscript{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\textsuperscript{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\textsuperscript{12} and multilocus typing to identify \textit{R. solanacearum} from wilted host plants.

\section*{2.3 Materials and methods}

\subsection*{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).
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 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)\textsuperscript{12} multiplex PCR was performed using five different phylotype specific primers:

(i) \textit{Nmult: 21:1F: CGTTGATGAGGCGCGCAATTT;}

(ii) \textit{Nmult: 21:2F: AAGTTATGGACGGTGGAAGTC;}

(iii) \textit{Nmult: 23: AF: ATTACSAGAGCAATCGAAAGATT;}

(iv) \textit{Nmult:22:InF: TTGCCAAGACGAGAAGAAGTA;}

(v) \textit{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\textsubscript{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’-AAGGAGGT TGATCCAGGCC-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 GAGCGGATTTGCGCTTGGTG (oRK002), RSp0540: ATGGACAGCGCGGCCTTGAC (oRK007) and GGGCGGAC AAAGTTG (oRK008); CAGCGTCAACATCGGCGGGT (oRK009), TGCCGCT CGCATTGGTCTGG (ORK010), no amplification occurs using this pair (oRK009 and oRK010) of primers (Fig. 2.4), RSp1071: TCACGGATGGCGCAAGCAG
(oRK013), and CGCCCGGCATCAAATGCATCC (oRK014); RSp1073: CGGT CAACAACAGCGCGTC (oRK019), and CGTGCTGTCTTGCGCCAGTT (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)\textsuperscript{28}. F1C1 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\textsuperscript{-1}: FeSO\textsubscript{4}·7H\textsubscript{2}O, 1.25 x10\textsuperscript{-4}; (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 0.5; MgSO\textsubscript{4}·7H\textsubscript{2}O, 0.05; KH\textsubscript{2}PO\textsubscript{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 wilting 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.

![Image of ooze test]

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\(^\text{12}\). 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\(^\text{12}\). 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)\(^\text{12}\) 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.

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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 \textit{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 \textit{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 \textit{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: F1C1 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 \textit{R. solanacearum} strains\textsuperscript{33}.

2.4.4 Pathogenicity test of F1C1

\textit{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 \textit{R. solanacearum} is intriguing. Though we had collected the ooze from the infected plant after surface sterilization, bacteria we isolated along with \textit{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 \textit{R. solanacearum} in plants followed by localization study in plants will prove their association with \textit{R. solanacearum} during infection.

\section*{2.5 Discussion}

In this work, we identified a \textit{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 \textit{R. solanacearum} spp. F1C1 belongs to phylotype I of \textit{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\textsuperscript{12}.

In the pathogenicity experiment we did not observe 100 \% wilting in all the infected plants. This is a usual observation in \textit{R. solanacearum} infection study (one of us has personal experience while working with \textit{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 \textit{R. solanacearum} can grow inside resistant \textit{Arabidopsis thaliana} without causing wilting\textsuperscript{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. 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.
2.6 References


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