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

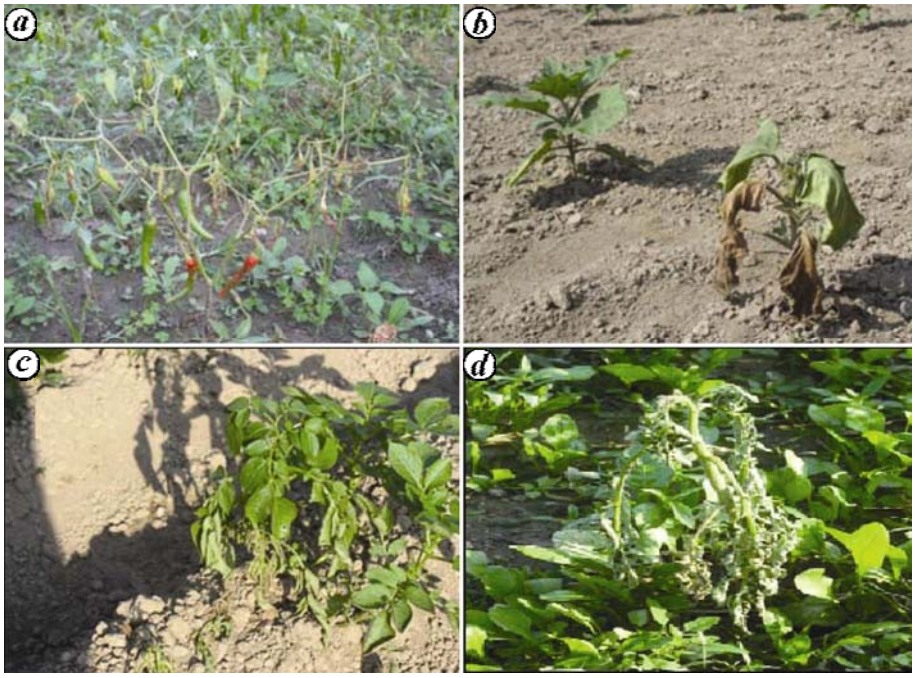

Figure 1. Wilted plants from different crop fields. ***a***, Wilted chilli plant. ***b***, Wilted potato plants along with some healthy potato plants. ***c***, Wilted brinjal plant (egg plant) along with a healthy brinjal plant in the same field. ***d***, Wilted tomato plant.

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.

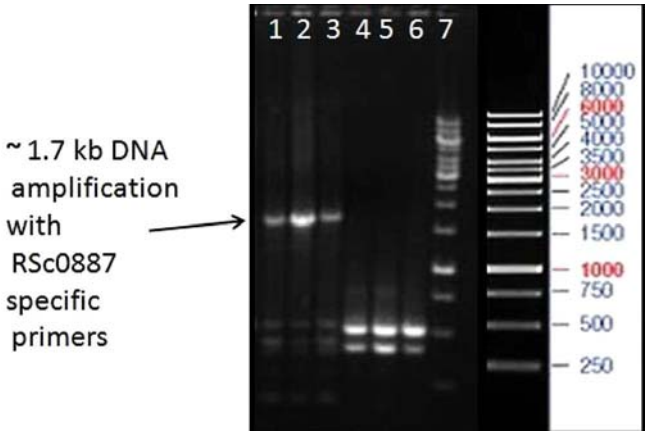

Figure 2. Gel photographs demonstrating amplification of a desired size DNA in FICI genome using oligo designed against RSc0887 locus of GMI1000 genome. Lane 7, 1 kb gene ruler (Fermentas, UK). The size of different DNA markers is given by the side. Lanes 1–3, Amplification of ~1.7 kb DNA from RSc0887 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

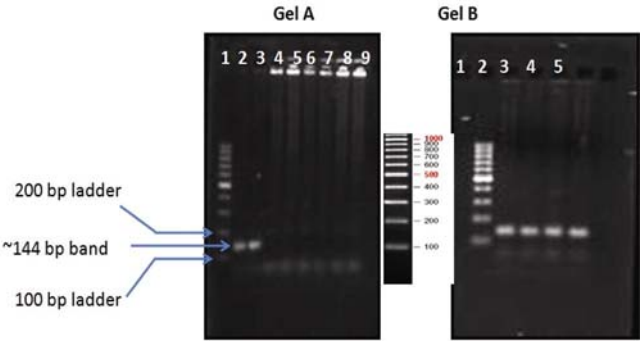

Figure 3. Gel photograph of multiplex PCR of various bacterial isolates. ***a***, Lane 1, Gene ruler (Fermentas, UK). The size of the markers from the lower to upper end of the gel is 100, 200, 300 bp, etc. Lanes 2, 3, Amplification of the phylotype-I-specific ~144 bp in F1C1. Lanes 4–9, No amplification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

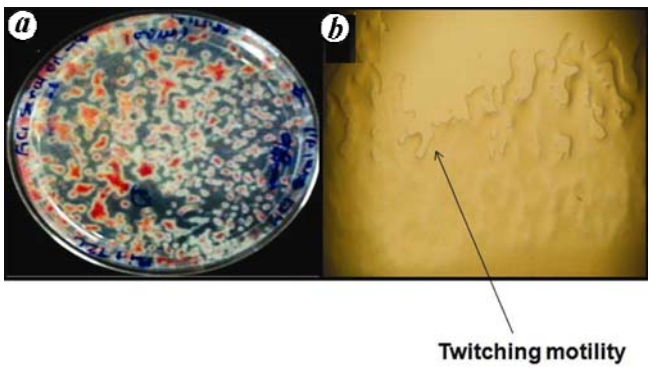

Figure 4. FIC1 growth on Phi plate. (***a***) On TZC-containing Phi plate F1C1 forms pinkish centred colony having white periphery. (***b***) Twitching motility in F1C1. Finger-like projections (called twitching motility) from the edges of the bacterial growth are observed after 24 h streaking on Phi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

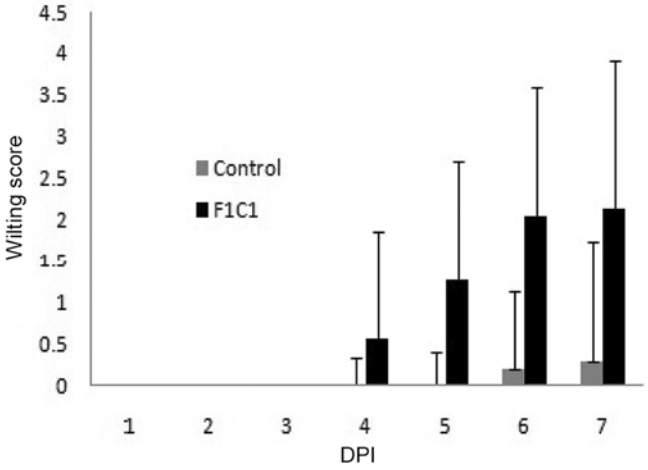

Figure 5. Wilting score till seven days post-inoculation. Totally 40 plants were inoculated with F1C1 by stem pricking and 20 plants were inoculated with sterile water by stem pricking. Wilting was scored using a scale 0.0 (for no or 0% wilting) to 4.0 (for 100% wilting). First wilting symptoms appeared 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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