Characterization of polyphosphate metabolism homologues of *Ralstonia solanacearum* F1C1 with regard to their role in virulence

4.1 Abstract

Polyphosphate (PolyP), a linear polymer of inorganic phosphate residues linked by high energy phospho-anhydride bonds is present in all living organisms and genes such as *ppk1*, *ppk2*, *ppx* and *ppnk* are known to be involved in polyphosphate metabolism in different organisms. *R. solanacearum* is a destructive plant pathogenic bacterium and leading cause of bacterial wilt disease in wide range of hosts. All the above four genes involved in PolyP metabolism are present in the genome of this bacterium and role of none of the genes has been characterized in this bacterium. We genetically characterized these four genes of PolyP metabolism by creating individual insertion mutations and studied their virulence phenotypes. We observed reduced swimming motility in case of *ppk1, ppk2* and *ppnk* mutants while *ppx* mutant was like the wild type *R. solanacearum* F1C1. The four mutant strains were proficient for twitching motility, extracellular cellulase activity and hypersensitive response in tobacco leaves. With regard to their virulence aspects, *ppk1* and *ppnk* insertion mutants were found to be significantly deficient for virulence when inoculated in tomato seedlings. Virulence deficiency observed was not that significant in case of *ppk2 and ppx* mutants. Expression of all the PolyP metabolism homologues was studied by measuring β-galactosidase activity expressed under the control of respective gene promoters. We also studied their real-time gene expression profile in wild type F1C1. We believe, this is the first initiative towards characterization of these genes in any broad host range plant pathogenic bacterium.

4.2 Introduction

During evolution, microorganisms have adapted the ability to reserve metabolites important for their viability and metabolic functioning. Best example is inorganic polyphosphate (PolyP) which is an important metabolite conserved amongst microorganisms. Polyphosphate (PolyP) is an un-branched polymer of 3 to several hundred phosphate residues linked with each other by high energy phospho-anhydride bonds [1]. The role of this molecule has been studied in different bacteria. It is a

multifunctional compound playing important role in many structural and physiological functions in cells. Moreover, this molecule has vital role in stationary-phase adaptation and stress tolerance of microorganisms, well reported in *E. coli*. PolyP metabolism involves several enzymes, among which polyphosphate kinase (PPK) and exopolyphosphatase (PPX), which are the principal enzymes responsible for the synthesis and the breakdown of PolyP in bacteria, respectively. Previous investigations revealed the role of *ppk* in motility of many bacteria such as *E. coli, Pseudomonas aeruginosa, Vibrio cholera, Helicobacter pylori, Salmonella spp., Klebsiella pneumonae* etc. [2,3,4,5] . In *P. aeruginosa, ppk* is needed for swimming, swarming and twitching motility and is involved in pathogenesis [3].

R. solanacearum, the second most destructive plant pathogenic bacterium, causes a lethal wilt in many plants, yielding heavy losses every year [6]. It possesses PolyP metabolism genes such as *ppk1, ppk2, ppx* and *ppnk*. These genes are conserved across phylotypes (Table 4.4) suggesting some important role to play in this pathogen. However, none of the PolyP metabolism genes has been characterized in this bacterium. The bacterium also exhibits different twitching and swimming motilities like in *P. aeruginosa*. Since, motility is an important function for *R. solanacearum* pathogenesis, a possible role of PolyP in motility of *R. solanacearum* and hence in its virulence cannot be eliminated.

In *R. solanacearum* GMI1000 strain, the features of the *ppk1, ppk2, ppx* and *ppnk* genes have been annotated as follows:

Gene	Nucleotide (bp)	Amino acid	Feature
RSc1536 (ppk1)	2214	737	Polyphosphate kinase
RSc0138 (*ppk2)*	828	275	Polyphosphate kinase 2
RSc1537 (*ppx)*	1584	527	Exo-polyphosphatase
RSc2650 (*ppnk)*	909	302	Inorganic polyphosphate /ATP-NAD kinase

The genes encoding PolyP synthesizing enzymes, *ppk1* and *ppk2* are distantly located in the chromosome. The BLASTn analysis reveals no significant sequence similarity between them, while they share 33% identity at their protein level. The exoployphosphatase gene, *ppx* is present downstream to the *ppk1* in a reverse

direction. The *ppnk* gene, encoding one of PolyP utilizing enzymes is also located on chromosome probably in an operon with *recN* gene (Fig.4.1).

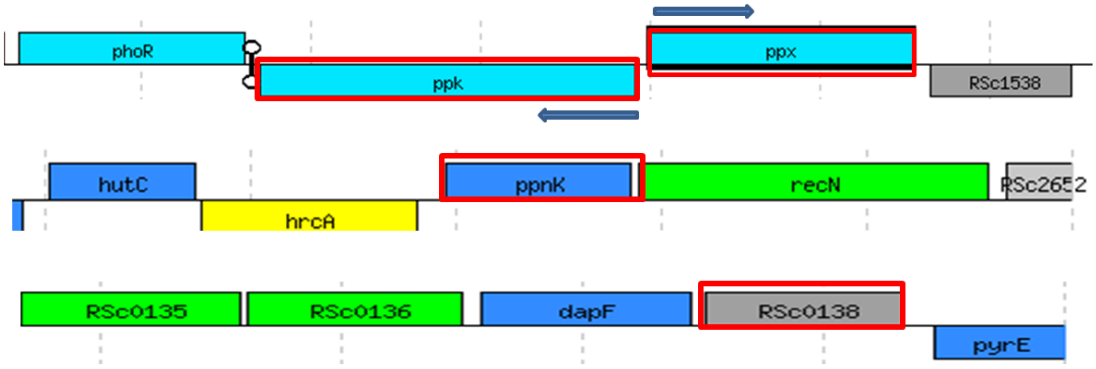


Fig.4.1: Arrangement of polyphosphate metabolism genes in *R. solanacearum* GMI1000 chromosome (Source: https://iant.toulouse.inra.fr/bacteria/annotation/cgi/ralso.cgi.)**.**

We are working on F1C1 strain of *R. solanacearum* [7], a phylotype I strain which has been already sequenced by us (unpublished). All the four polyphosphate metabolism gene homologues are present in this strain. In this study we have characterized the four genes with regard to their role in virulence using tomato seedlings as a model host.

4.3 Material and methods

4.3.1 Media and chemicals

Bacterial growth media components, chemicals and antibiotics used were purchased from Hi-Media, Mumbai, India. All enzymes including Dream Taq polymerases, restriction endonucleases, T4 ligases, dNTPs, CDNA synthesis kit, Maxima SYBR Green/ROX qPCR Master Mix and DNA ladders (Generuler) were procured from Fermentas (Thermo Fisher Scientific, Mumbai, India). Genomic DNA isolation kits, Plasmid DNA isolation kits, Gel-extraction kits were bought from Qiagen (New Delhi, India) and Nitrocellulose membranes (Amersham Protran, 0.45 µm NC) were bought from GE Healthcare, Germany. All Plastic wares and Glasswares were purchased from Tarsons, Kolkata, India and Borosil, Kolkata, India, respectively. All synthesized primers used in this study were obtained from Bioserve (Hyderabad, India).

4.3.2 Bacterial strains, growth media and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 4.1. Wild type *R. solanacearum* F1C1and derivative mutants were grown in BG medium (Bacto peptone, 10 g/l; Casamino acids, 1 g/l; Yeast extract, 1 g/l) at 28°C in an incubator (Orbitek, Scigenics Biotech, India) for 48 hr. To 200 ml of BG medium, 5 ml of 20 % glucose were added. *E. coli*- DH5α strain was grown in LB (20.0 g/l) medium at 37°C. Agar (15.0 g/l) was added in case of solid medium. Concentration of different antibiotics used in the experiments was as: Nalidixic acid (Nal; 50µg/ml), Ampicillin (Amp; 50µg/ml), spectinomycin (Spc; 50µg/ml) and Gentamycin (Gen; 50µg/ml).

Table 4.1: List of plasmids and bacterial strains used in this study

Plasmids/ strains	**Relevant characteristics**	**Reference**
Plasmids and *Escherichia coli* strain		
pGEMT	Amp^r, Cloning vector	Promega
pTZ57R/T	Amp^r, Cloning vector	Thermo Scientific
pCZ367	Amp^r, Gen^r, Insertional vector with *lacZ* reporter	
pTP001	Amp^r, pGEM-T-easy plasmid containing a region of *ppk1* amplified using oFppk1 and oRppk1 primer pair	This study
pTP002	Amp^r, Gen^r, pCZ367 plasmid containing the region of *ppk1* digested out from pTP001	This study
pTP003	Amp^r, pGEM-T plasmid containing a region of *ppk1* amplified using oFppk1.A and oRppk1.A primer pair	This study
pTP004	Amp^r, Spc^r, derived from pTP003 after insertion of ΩSpc cassette in *EcoRI* site of *ppk1* coding region	This study
pTP005	Amp^r, pTZ57R/T plasmid containing a region of *ppk2* amplified using oFppk2 and oRppk2 primer pair	This study
pTP006	Amp^r, Gen^r, pCZ367 plasmid containing the region of *ppk2* digested out from pTP005	This study
pTP007	Amp^r, pTZ57R/T plasmid containing a region of *ppx* amplified using oFppx and oRppx primer pair	This study
pTP008	Amp^r, Gen^r, pCZ367 plasmid containing the region of *ppx* digested out from pTP007	This study

pTP009	Amp^r, pGEM-T-easy plasmid containing a region of *ppnk* amplified using oFppnk and oRppnk primer pair	This study
pTP010	Amp^r, Gen^r, pCZ367 plasmid containing the region of *ppnk* digested out from pTP009	This study
pNP267	Amp^r, Gen^r, Chl^r , pRCG-GWY-based plasmid	[8]
pTP011	Amp^r, Gen^r, pNP267 plasmid containing full length *ppk1*amplified using oFppk3 and oRppk3 primer pair	This study
pTP012	Amp^r, Gen^r, Spc^r, derived from pTP011 after insertion of ΩSpc cassette in *HindIII* site of the plasmid	This study
pTP013	Amp^r, Gen^r, pNP267 plasmid containing full length *ppnk* amplified using oFppnk3 and oRppnk3 primer pair	This study
pTP014	Amp^r, Gen^r, Spc^r, derived from pTP013 after insertion of ΩSpc cassette in *HindIII* site of the plasmid	This study
DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A*sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Lab strain
***Ralstonia solanacearum* strains**		
F1C1	Wild type virulent *R. solanacearum*	Lab strain [7]
TRS1019	*ppk1*::pCZ367; Amp^r, Gen^r, *ppk1* deficient, swimming motility deficient, Vir^-, derived from F1C1	This study
TRS1020	*ppk1*::ΩSpc; Spc^r, *ppk1* deficient, swimming motility deficient, Vir^-, derived from F1C1	This study
TRS1021	*ppk2*::pCZ367; Amp^r, Gen^r, *ppk2* deficient, swimming motility deficient, Vir^-, derived from F1C1	This study
TRS1022	*ppx*::pCZ367; Amp^r, Gen^r, *ppx* deficient, swimming motility proficient, Vir^-, derived from F1C1	This study
TRS1023	*ppnk*::pCZ367; Amp^r, Gen^r, *ppnk* deficient, swimming motility deficient, Vir^-, derived from F1C1	This study
TRS1024	*ppk1*:: ΩSpc::*ppk2*::pCZ367; Amp^r, Gen^r, Spc^r, *ppk1*& *ppk2* deficient, swimming motility deficient, Vir^-, derived from F1C1	This study
TRS1025	*ppk1*:: ΩSpc::pNP267; Amp^r, Gen^r, Spc^r, *ppk1* complemented, Vir^-, derived from *ppk1* insertion mutant	This study
TRS1026	*ppnk*:: ΩSpc::pNP267; Amp^r, Gen^r, Spc^r, *ppnk* complemented, Vir^-, derived from *ppnk* insertion mutant	This study

4.3.3 Competent cell preparation and transformation

E. coli DH5α competent cells were prepared by using PIPES following Inoue et al., (1990) protocol and competent cells were transformed by heat-shock at 42°C for 90 sec [9].

Competent cell preparation and natural transformation in *R. solanacearum* F1C1were performed as described earlier in chapter 2.

4.3.4 Identification of polyphosphate metabolism homologues in *R. solanacearum* F1C1 strain

Recently, whole genome sequencing of F1C1 strain of *R. solanacearum* has been completed in our laboratory (unpublished work). At the beginning of this work, annotation of the sequenced genome has not been completed. Therefore, to identify the presence of polyphosphate gene homologues namely *ppk1*, *ppk2*, *ppx* and *ppnk* in F1C1, we took reference gene sequence from GMI1000 strain belonging to phylotype I. Nucleotide sequences of the genes were obtained from website (https://iant.toulouse.inra.fr/bacteria/annotation/cgi/ralso.cgi.), GMI1000 genome database.

We took the sequences from GMI1000 and found out corresponding gene sequences in F1C1 genome by homology search performed using the NCBI blast tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.3.5 Creation of insertion mutagenesis in polyphosphate metabolism homologues of *R. solanacearum* F1C1

After the identification of polyphosphate metabolism homologues in F1C1 genome, we characterized these genes by insertion mutagenesis. We created individual insertion mutation in *ppk1*, *ppk2*, *ppx* and *ppnk* homologues of F1C1 by *lacZ* reporter gene fusion method. In Fig.4.2, schematic representation of the methodology has been shown.

We designed the primers for all four genes using Primer-BLAST-tool (NCBI). For all the genes, at the 5’ end of forward primer, *HindIII* restriction site (5’-AAGCTT-3’) and at the 5’ end of the reverse primer *XbaI* site (5’-TCTAGA-3’) was incorporated during primers designing. All the primers used for the characterization study has been listed in Table 4.2.

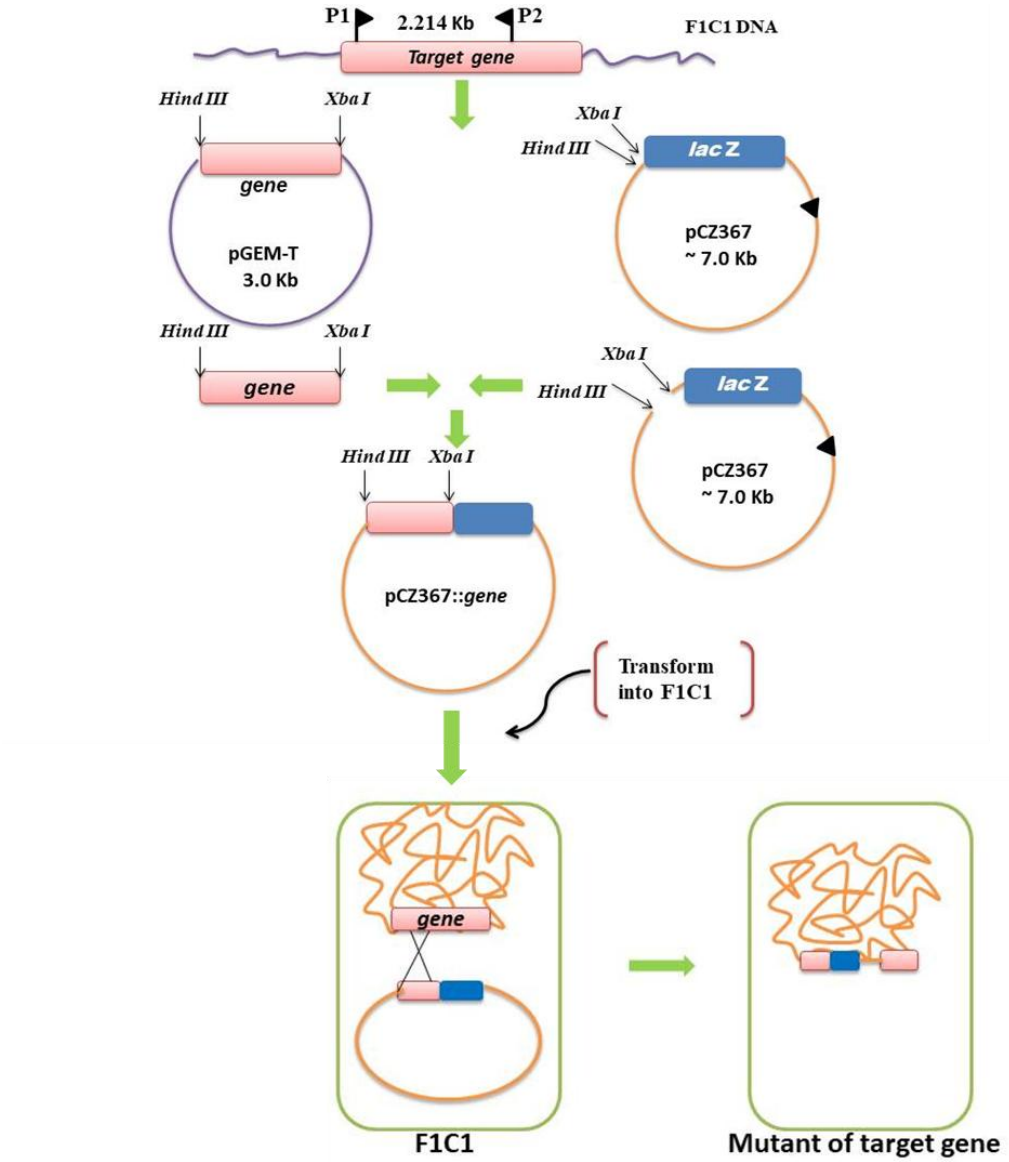


Fig.4.2: Schematic representation of *lacZ* reporter gene fusion strategy by single homologous recombination event used to construct insertion mutagenesis.

We used genomic DNA of F1C1 as template for amplification and PCR conditions as well as annealing temperatures of all the genes were standardized. For amplification of all the genes, each PCR reaction was set in 20 µl reaction volume consisting of 2.0 µl of 10X Dream Taq buffer, 1.2 µl of 2 mM dNTP mix, 0.9 µl of DMSO, 0.2 µl of Dream-Taq DNA polymerase (5U/µl), 1.0 µl of 5 µM forward primers and 1.0 µl of 5 µM reverse primer. Final volume was adjusted to 20 µl with Nuclease-free water.

The amplification was performed on a PCR machine (Eppendorf Mastercycler Nexus, Germany). Amplified products of all the genes were visualized on 0.8% agarose gels (1xTAE, 70V, 200 mA & 35 min) and observed in a gel-doc system (E-gel imager, Thermo-Fisher Scientific, Mumbai, India). Subsequently, amplified DNA products were gel extracted using Qiagen gel extraction kit and sequenced at Molbiogen (Guwahati, India).

Table 4.2: List of primers used in the characterization study of PolyP homologues

Sl. No	Name of the primer	Sequence	Remarks
1	oFppk1	5'-GCCAAGCTTGCCGGATAGTCATTCGTTGGA-3'	*ppk1* (amplicon, used for creating *lacZ* insertion mutation)
2	oRppk1	5'-GCCTCTAGAACTTCAGCATCGGGCGG-3'	
3	olacR1	5'-AAGGGGGATGTGCTGCAAGG-3'	*lacZ* confirmation in pCZ367
4	oFppk4	5'-GCCAAGCTTGGCCGGATAGTCATTCGTTG-3'	used for confirmation of *ppk1* insertion
5	oSpc5-01	5'- CGTTACCACCGCTGCGTTCGG-3'	Ω-Spc cassette confirmation
4	oFppk1.A	5'-GCCCCTGCTCAATCGGGAAGTCG-3'	*ppk1* (amplicon, used for creating Ω-Spc insertion mutation
5	oRppk1.A	5'-GCCGCGAGAAGTGGTTCAGCAGAT-3'	
6	oFppk3	5'-GCCGGTACCAAGCTGTTGGAACCCATGTC-3'	*ppk1* (amplicon, used for complementation study)
7	oRppk3	5'-GCCAAGCTTCTTTTCCGCCCTTCAAGC-3'	
8	oFppk2	5'-GCCAAGCTTATGGGATCGAACGGCAAGAC-3'	*ppk2* (amplicon, used for creating *lacZ* insertion mutation)
9	oRppk2	5'-GCCTCTAGACATTCGATCTTCACCACCTCA-3'	

10	oFppx	5'-GCCAAGCTTCATCACAATCCCCCTGCGTA-3'	*ppx* (amplicon, used for creating *lacZ* insertion mutation)
11	oRppx	5'-GCCTCTAGAGATGCTCATGCCGATCTCGT-3'	
12	oFppnk	5'-GCCAAGCTTGCCACTTCGCCCTTCAAGAC-3'	*ppnk* (amplicon was used for creating *lacZ* insertion mutation)
13	oRppnk	5'-GCCTCTAGAACAGCGCATAGGCCGTC-3'	
14	oFppnk3	5'-GCCGGTACCCCGGAATACTTCGACAAGGT-3'	*ppnk* (amplicon, used for complementation study)
15	oRppnk3	5'-GCCAAGCTTATCGGTTTTTGGCGTTACAG-3'	
16	oNP611	5'-GAAAATTCCCCCGTTCCTGGT-3'	pNP267 (amplicon, used to confirm complementation vector)
17	oNP612	5'-CCGAGTATATTCACGGGGCTT-3'	

4.3.5.1 Creation of ppk1insertion mutant

For *ppk1* homologue, PCR conditions were standardized as: (step1) initial denaturation at 95°C for 5 min; (step 2 for 35 cycles) denaturation at 95°C for 30 sec; annealing at 66.2°C for 30 sec; extension at 72°C for 1 min and (step3) final extension at 72°C for 7 min. The primer set [oFppk1 and oRppk1] showed successful amplification of ~977 bp *ppk1*gene fragment (Fig.4.3). The amplified fragment was then ligated to cloning vector pGEM-T-easy (Promega, New Delhi, India) following manufacturer's recommendations. Ligated product was then transformed into competent *E. coli* DH5α cells and selected on LB-agar plate supplemented with ampicillin antibiotic. Recombinant plasmid pTP001 (pGEM-T-easy::*ppk1*) was isolated from selected transformant using Qiagen plasmid isolation kit and subsequently confirmed by restriction digestion. The plasmid pTP001 was digested with *HindIII* and *XbaI* restriction enzymes simultaneously to release the ~977 bp size fragment containing *HindIII* and *XbaI* digested sticky ends at 5' and 3' end respectively (Fig.4.4).

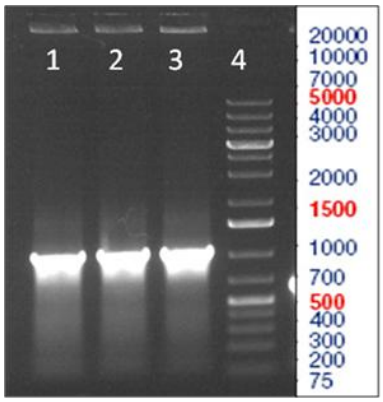


Fig.4.3: Agarose gel showing partial amplification of *ppk1* homolog in F1C1 genome. The ~977 bp fragment amplified with oFppk1 and oRppk1 primers using F1C1 genomic DNA as template. Lane 1, 2 and 3 shows ~ 977 bp amplicon of *ppk1* homologue. Lane 4 has 1 kb plus DNA ladder. The size of different DNA bands is showing at the side of the gel.

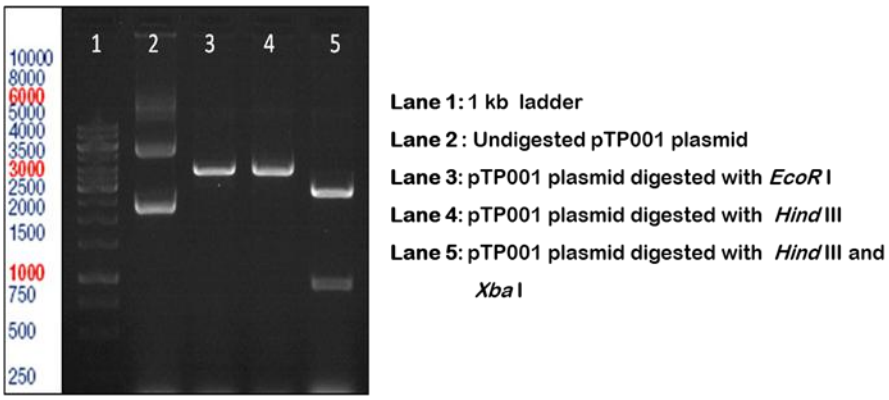


Fig.4.4: Agarose gel showing cloning of *ppk1* gene fragment in pGEM-T-easy vector. Lane 1 has 1 kb DNA ladder. Lane 2 shows undigested pTP001 plasmid. Lane 3 and 4 show restriction digestion of pTP001 plasmid with *EcoRI* and *HindIII*, respectively yielding ~4.0 kb size linear constructs. Lane 5 shows double digestion of pTP001 plasmid with *HindIII* and *XbaI* yielding a ~977 bp size *ppk1* fragment and a ~3.0 kb size pGEM-T-easy vector. The size of different DNA bands is showing at the side of the gel.

The double digested *ppk1* fragment was then ligated to plasmid promoterless insertional vector, pCZ367 [10] digested in a similar way with *HindIII* and *XbaI* enzymes. Ligation step was performed at 4°C following standard protocols. Ligated product obtained was then transformed to competent DH5α cells and recombinants showing resistant against ampicillin and gentamycin antibiotics were selected. Recombinant plasmid pTP002 (pCZ367::*ppk1*) was isolated from *E. coli* and was confirmed by double digestion with *HindIII* and *XbaI* (Fig.4.5). In every step of cloning, recombinant plasmids were also confirmed with PCR amplification of the insert gene fragment.

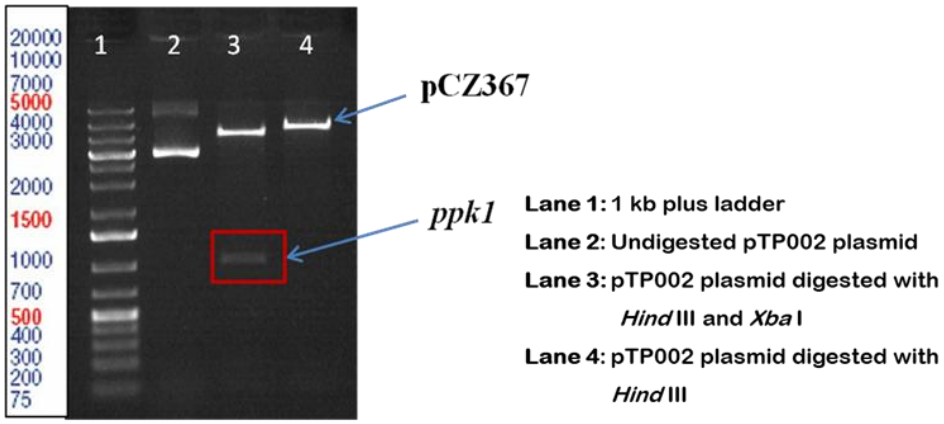


Fig.4.5: Agarose gel showing cloning of *ppk1* gene fragment in pCZ367 vector. Lane 1 has 1 kb plus DNA ladder. Lane 2 shows undigested pTP002 plasmid. Lane 3 shows double digestion of pTP002 plasmid with *EcoRI* and *HindIII* enzymes yielding ~977 bp size *ppk1* fragment and ~7.0 kb size pCZ367 plasmid. Lane 4 shows restriction digestion of pTP002 plasmid with *HindIII*, yielding ~8.0 kb size linear vector. The size of different DNA bands is showing at the side of the gel.

For natural transformation of *R. solanacearum* F1C1, methodology described by Gonzalez, et al. (2011) [11] was followed with modifications in glycerol concentration. We used 10% glycerol in Minimal Medial to make the bacterium competent. Competent F1C1 was transformed with recombinant plasmid pTP002 and successful transformants were selected on BG-agar plate containing ampicillin and gentamycin. The F1C1 transformants harboring pTP002 plasmid by the single homologous recombination event was confirmed by PCR amplification. For this confirmation, a reverse primer olacR1[10] at the 5' end of the *lacZ* gene and forward primer oFppk4 at the 5' end upstream of the *ppk1* gene sequences used to create the insertion mutant were designed. Amplification of ~1200 bp size DNA band in F1C1 transformants indicated successful insertion event leading to the creation of *ppk1* mutant strain of F1C1 (Fig.4.6). TRS1019 strain was used for further studies.

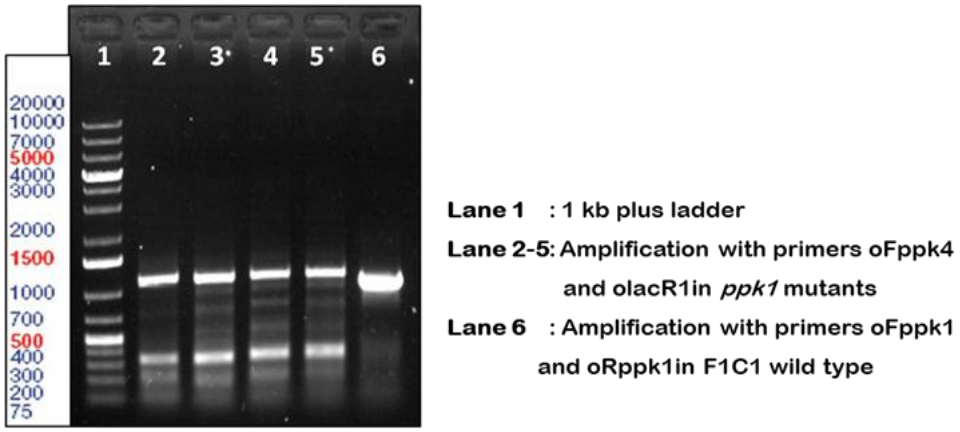


Fig.4.6: Agarose gel showing insertion mutation in *ppk1* homologue of F1C1. Lane 1 has 1 kb plus DNA ladder. Lane 2, 3, 4 and 5 show PCR amplification with primers oFppk4 and olacR1 in *ppk1* insertion mutants yielding ~1200 bp size DNA band. Lane 6 shows PCR amplification with primers oFppk1 and oRppk1 in wild type F1C1 yielded ~977 bp DNA band. This confirmed insertion is indeed at *ppk1* homologue of F1C1. The size of different DNA bands is showing at the side of the gel.

4.3.5.2 Creation of ppk2 insertion mutant

We created *ppk2* insertion mutant of F1C1 in a similar way following the steps used for *ppk1* mutant creation. The primer set [oFppk2 and oRppk2] showed successful amplification of ~827 bp *ppk2* gene fragment (Fig.4.7) with following PCR reaction conditions:

Initial denaturation at 95°C for 5min	(step1for 1 cycle)
Denaturation at 95°C for 30 sec Annealing at 62°C for 30 sec Extension at 72°C for 1min	(step 2 for 35 cycles)
Final extension at 72°C for 7 min	(step3 for 1 cycle)

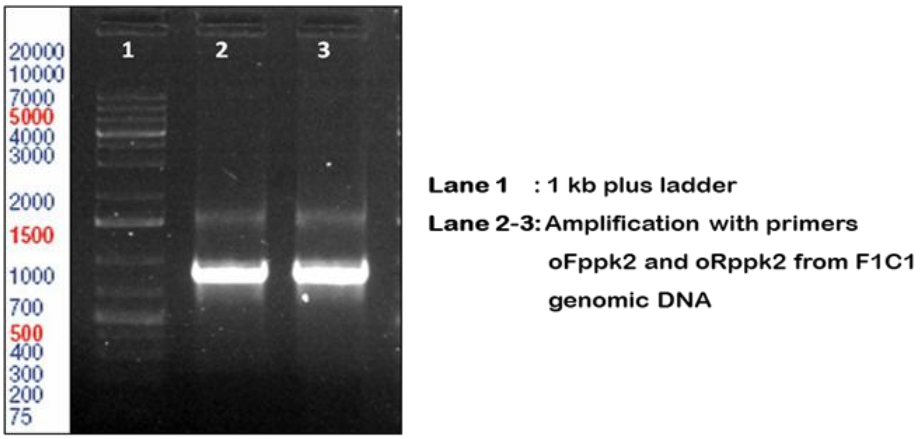


Fig.4.7: Agarose gel showing partial amplification of *ppk2* homologue in F1C1 genome. The ~827 bp fragment amplified with oFppk2 and oRppk2 primers using F1C1 genomic DNA as template. Lane 1 has 1 kb plus DNA ladder. Lane 2 and 3 show ~827 bp amplicon of *ppk2* homologue. The size of different DNA bands is showing at the side of the gel.

The *ppk2* amplified fragment was then cloned in pTZ57R/T vector and subsequently sub-cloned into pCZ367 vector using *EcoRI* and *HindIII* restriction sites. The confirmatory gel pictures have been shown in Fig.4.8 and Fig.4.9, respectively.

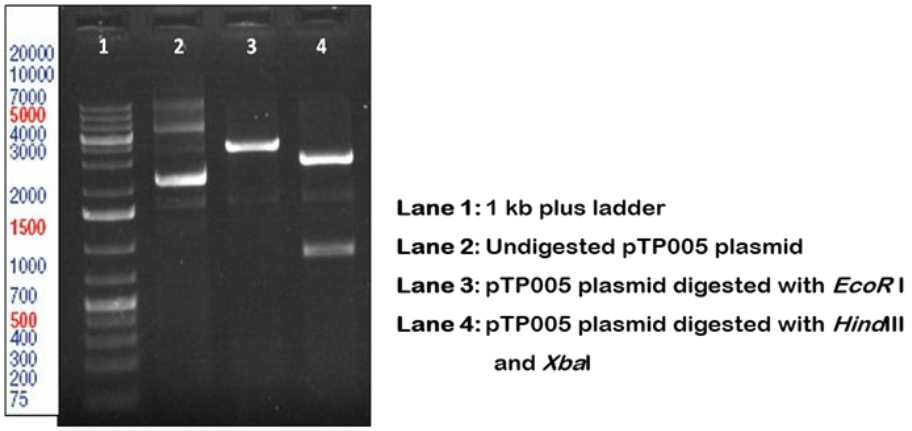


Fig.4.8: Agarose gel showing cloning of *ppk2* gene fragment in pTZ57R/T vector. Lane 1 has 1 kb plus DNA ladder. Lane 2 shows undigested pTP005 plasmid. Lane 3 shows restriction digestion of pTP005 plasmid with *EcoRI* yielding a ~3.8 kb size linear plasmid. Lane 4 shows double digestion of pTP005 plasmid with *HindIII* and *XbaI* yielding a ~827 bp size *ppk2* fragment and a ~2.9 kb size pTZ57R/T vector. The size of different DNA bands is showing at the side of the gel.

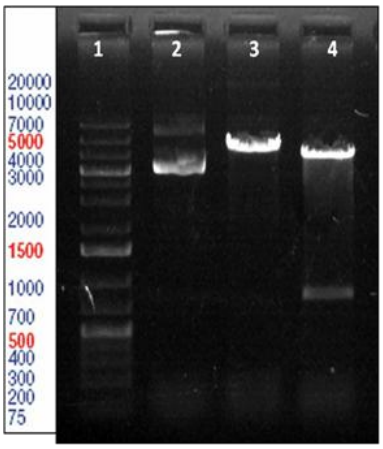


Fig.4.9: Agarose gel showing cloning of *ppk2* gene fragment in pCZ367 vector. Lane 1 has 1 kb plus DNA ladder. Lane 2 shows undigested pTP006 plasmid. Lane 3 shows restriction digestion of pTP006 plasmid with *HindIII*, yielding ~7.9 kb size linear vector. Lane 4 shows double digestion of pTP006 plasmid with *HindIII* and *XbaI* enzymes yielding ~827 bp size *ppk2* fragment and ~7.0 kb size pCZ367 plasmid. The size of different DNA bands is showing at the side of the gel.

After the confirmation of cloning of *ppk2* gene fragment in pCZ367 vector, we transformed F1C1 wild type with the recombinant plasmid, pTP006 and successful transformants were selected on BG-agar plate supplemented with ampicillin and gentamycin. The resulting *ppk2* insertion mutant of F1C1 was confirmed by PCR amplification with primer pair olacR1(reverse) designed at the 5' end of the *lacZ* gene and oFppk2 (forward), which was utilized for amplifying the initial ~827 bp DNA amplicon to create the insertion mutation. Amplification of ~1100 bp size DNA band in transformants demonstrated successful insertion of pTP006 in *ppk2* homologue leading to the creation of *ppk2* mutant strains of F1C1 (Fig.4.10). One of the *ppk2* mutants, TRS1021 strain was recruited in further studies.

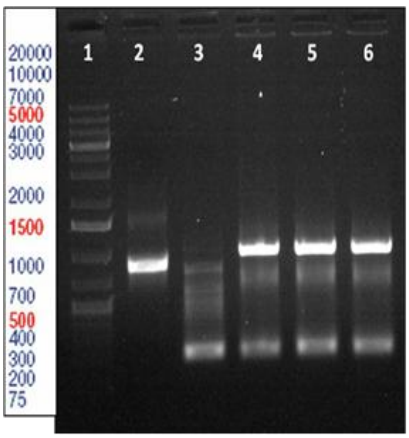


Fig.4.10: Agarose gel showing insertion mutation in *ppk2* homologue of F1C1. Lane 1 has 1 kb plus DNA ladder. Lane 2 shows PCR amplification with primers oFppk2 and oRppk2 in wild type F1C1 yielding ~827 bp DNA band. Lane 3 shows PCR amplification with primers oFppk2 and olacR1 in wild type F1C1 that yielded no such band as observed in case of mutants. Lane 4, 5 and 6 shows amplification with primers oFppk2 and olacR1 in *ppk2* insertion mutants yielding ~1100 bp size DNA bands. The size of different DNA bands is showing at the side of the gel.

4.3.5.3 Creation of ppx insertion mutant

Insertion mutant of *ppx* homologue in F1C1 was created in the same way as described above. PCR reaction conditions used to amplify *ppx* gene fragment were as follows:

Initial denaturation at 95°C for 5min	(step1for 1 cycle)
Denaturation at 95°C for 30 sec Annealing at 64.4°C for 30 sec Extension at 72°C for 1min	(step 2 for 35 cycles)
Final extension at 72°C for 7 min	(step 3 for 1 cycle)

We amplified ~1189 bp *ppx* gene fragment with primer set [oFppx and oRppx] (Fig.4.11).

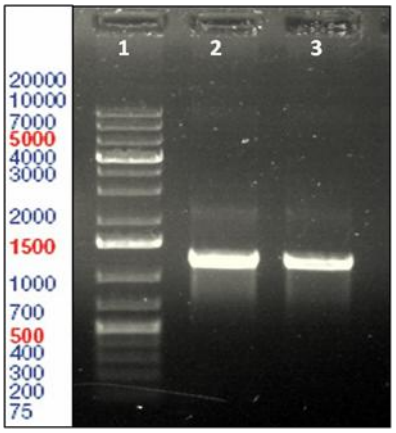


Fig.4.11: Agarose gel showing partial amplification of *ppx* homolog in F1C1 genome. The ~1189 bp fragment amplified with oFppx and oRppx primers using F1C1 genomic DNA as template. Lane 1 has 1 kb plus DNA ladder. Lane 2 and 3 show ~1189 bp amplicon of *ppx* homologue. The size of different DNA bands is showing at the side of the gel.

The confirmatory gel pictures showing cloning of amplified fragment in pTZ57R/T and pCZ367 vector has been shown in Fig.4.12 and Fig.4.13 respectively.

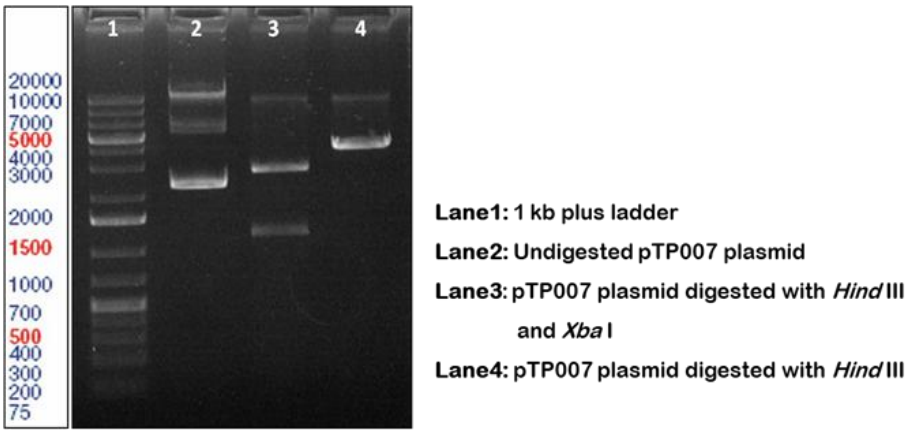


Fig.4.12: Agarose gel showing cloning of *ppx* gene fragment in pTZ57R/T vector. Lane 1 has 1 kb plus DNA ladder. Lane 2 shows undigested pTP007 plasmid. Lane 3 shows double digestion of pTP007 plasmid with *HindIII* and *XbaI* yielding a ~1189 bp size *ppx* fragment and a ~2.9 kb size pTZ57R/T vector. Lane 4 shows restriction digestion of pTP007 plasmid with *HindIII* yielding a ~4.1 kb size linear plasmid. The size of different DNA bands is showing at the side of the gel.

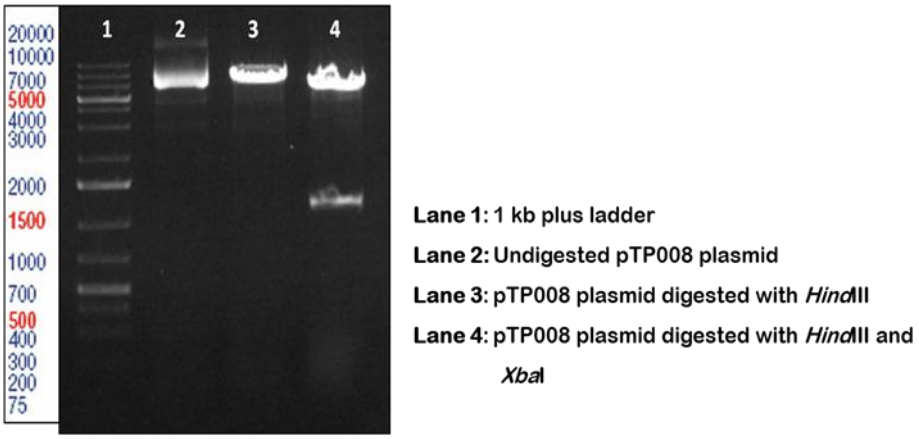


Fig.4.13: Agarose gel showing cloning of *ppx* gene fragment in pCZ367 vector. Lane 1 has 1 kb plus DNA ladder. Lane 2 shows undigested pTP008 plasmid. Lane 3 shows restriction digestion of pTP008 plasmid with *HindIII*, yielding ~8.2 kb size linear vector. Lane 4 shows double digestion of pTP008 plasmid with *HindIII* and *XbaI* enzymes yielding ~1189 bp size *ppx* fragment and ~7.0 kb size pCZ367 plasmid. The size of different DNA bands is showing at the side of the gel.

After the confirmation of positive clones, we transformed *R. solanacearum* F1C1 with pTP008 recombinant plasmid and successful transformants were selected and subsequently examined for *lac*Z insertion leading to the creation of *ppx* mutants. Mutant strain named TRS1022 was used for subsequent studies.

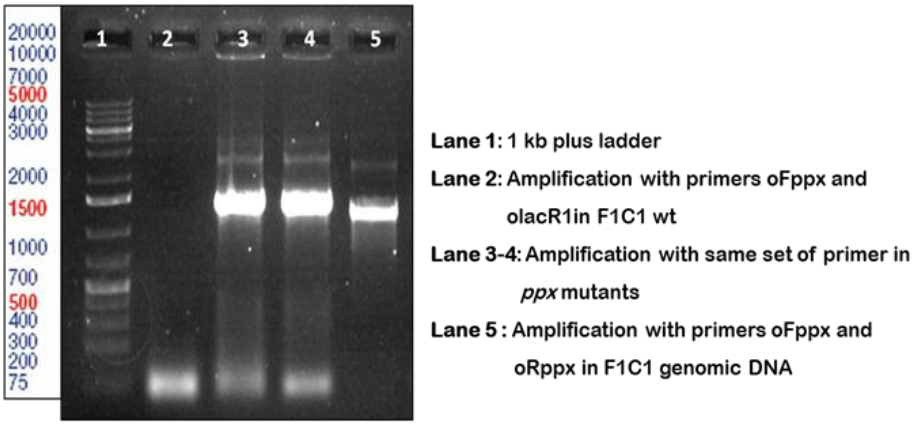


Fig.4.14: Agarose gel showing insertion mutation in *ppx* homologue of F1C1. Lane 1 has 1 kb plus DNA ladder. Lane 2 shows PCR amplification with primers oFppx and olacR1 in wild type F1C1 yielding no DNA band. Lane 3 and 4 show PCR amplification with primers oFppx and olacR1 in *ppx* insertion mutants that yielded ~1400 bp size DNA bands. Lane 5 shows amplification with primers oFppx and oRppx in wild type F1C1 yielding ~1189 bp size DNA band. The size of different DNA bands is showing at the side of the gel.

4.3.5.4 Creation of ppnk insertion mutant

In case of *ppnk*, the primer set [oFppnk and oRppnk] showed successful amplification of ~577 bp gene fragment (Fig.4.15) which was used for insertion mutant creation.

PCR reaction parameters for *ppnk* homologue were as:

Initial denaturation at 95°C for 5min	(step1for 1 cycle)
Denaturation at 95°C for 30 sec	(step 2 for 35 cycles)
Annealing at 66.2°C for 30 sec	
Extension at 72°C for 1min	
Final extension at 72°C for 7 min	(step3 for 1 cycle)

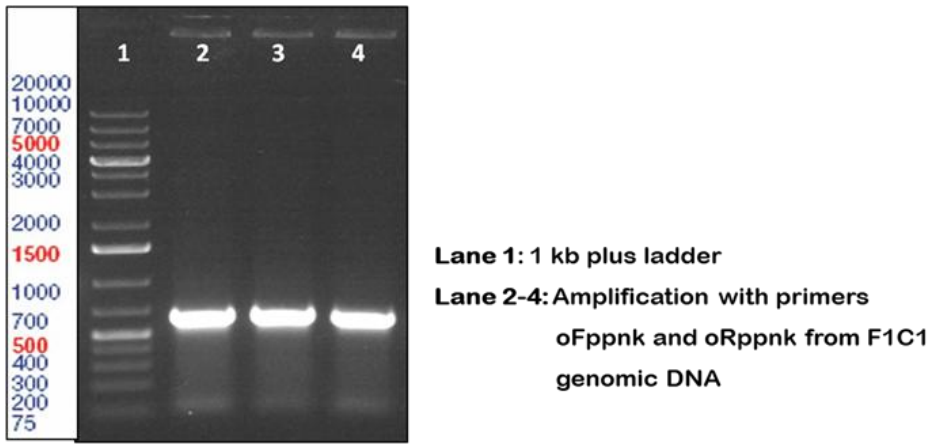


Fig.4.15: Agarose gel showing partial amplification of *ppnk* homologue in F1C1 genome. The ~577 bp fragment amplified with oFppnk and oRppnk primers using F1C1 genomic DNA as template. Lane 1 has 1 kb plus DNA ladder. Lane 2, 3 and 4 shows ~577 bp amplicon of *ppnk* homologue. The size of different DNA bands is showing at the side of the gel.

Amplified fragment of *ppnk* homologue was then cloned in pGEM-T-easy vector (Fig.4.16), sub-cloned in pCZ367 (Fig.4.17) and successfully transformed into F1C1 strain. The resultant transformant named TRS1023 was confirmed by PCR amplification with primer set olacR1, designed for *lac*Z and oFppnk and used for further characterization (Fig.4.18).

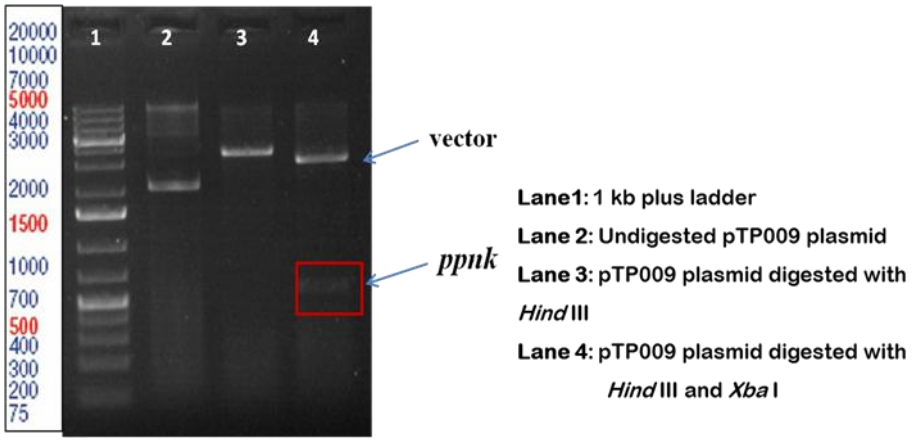


Fig.4.16: Agarose gel showing cloning of *ppnk* gene fragment in pGEM-T-easy vector. Lane 1 has 1 kb plus DNA ladder. Lane 2 shows undigested pTP009 plasmid. Lane 3 shows restriction digestion of pTP009 plasmid with *HindIII* yielding a ~3.6 kb size linear plasmid. Lane 3 shows double digestion of pTP009 plasmid with *HindIII* and *XbaI* yielding a ~577 bp size *ppnk* fragment and a ~3.0 kb size pGEM-T-easy vector. The size of different DNA bands is showing at the side of the gel.

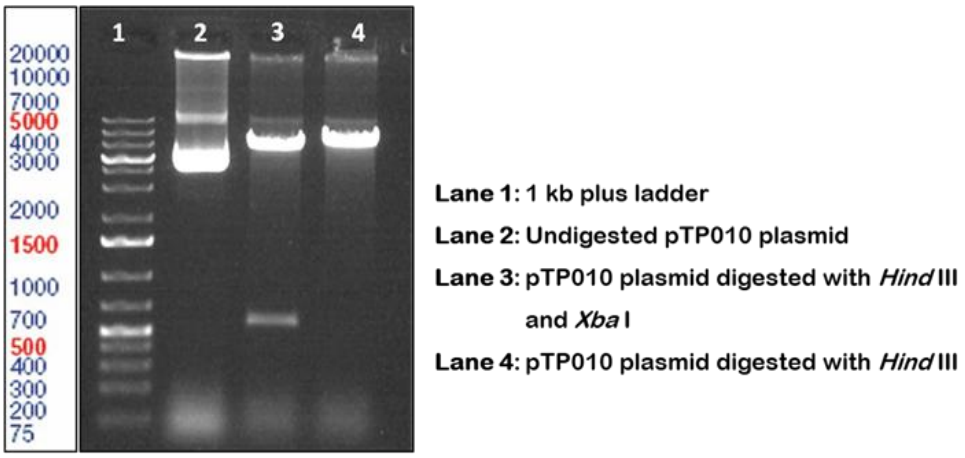


Fig.4.17: Agarose gel showing cloning of *ppnk* gene fragment in pCZ367 vector. Lane 1 has 1 kb plus DNA ladder. Lane 2 shows undigested pTP010 plasmid. Lane 3 shows double digestion of pTP010 plasmid with *HindIII* and *XbaI* enzymes yielding ~577 bp size *ppnk* fragment and ~7.0 kb size pCZ367 plasmid. Lane 4 shows restriction digestion of pTP010 plasmid with *HindIII*, yielding ~7.6 kb size linear vector. The size of different DNA bands is showing at the side of the gel.

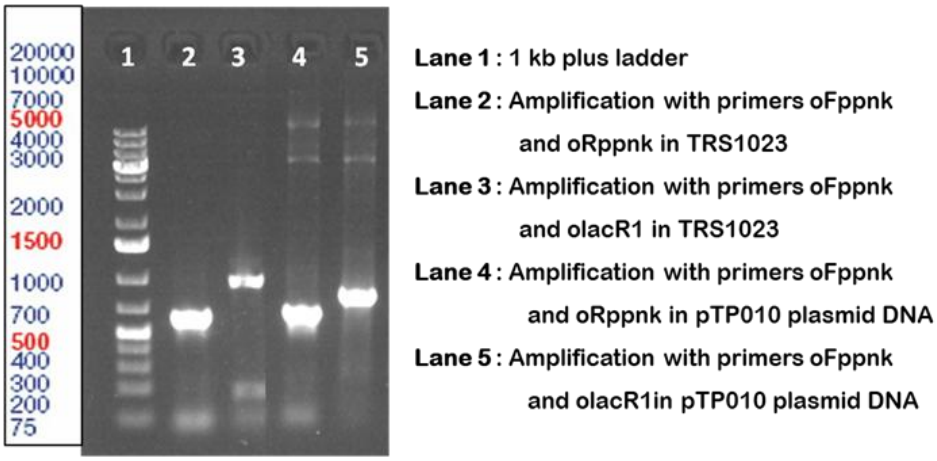


Fig.4.18: Agarose gel showing insertion mutation in *ppnk* homologue of F1C1. Lane 1 has 1 kb plus DNA ladder. Lane 2 and 4 show PCR amplification with primers oFppnk and oRppnk in *ppnk* insertion mutant and pTP010 plasmid yielding ~577 bp DNA bands. Lane 3 shows PCR amplification with primers oFppnk and olacR1 in *ppnk* mutant that yielded ~ 900 bp size DNA band. Lane 5 shows PCR amplification with same set of primers oFppnk and olacR1 in pTP010 plasmid that yielded ~ 800 bp size DNA band. The size of different DNA bands is showing at the side of the gel.

4.3.5.5 Assay for lacZ gene insertion in polyphosphate homologue mutants

lacZ gene insertion in *ppk1*, *ppk2*, *ppx* and *ppnk* mutants were further confirmed by X-gal assay. In a microfuge tube, 100 µl of milli-Q water (Millipore, Germany) was taken and a loop full of bacteria was added to make the bacterial

suspension. 2.0 µl of X-gal was added to it, mixed and incubated at 37°C for 30 min. Appearance of blue color in mutant colonies namely TRS1019, TRS1021, TRS1022 and TRS1023 indicated the insertion and hence expression of *lac*Z which was absent in wild type F1C1 suspension (Fig.4.19).

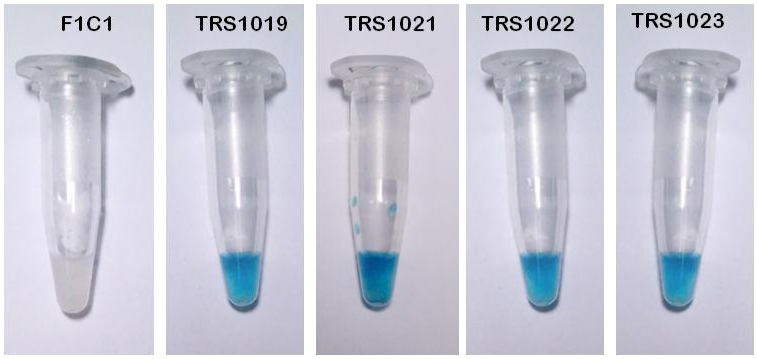


Fig.4.19: X-gal assay in polyphosphate homologue mutants of F1C1. Appearance of blue color in all insertion mutants (TRS1019, TRS1021, TRS1022 and TRS1023) confirmed the insertion of *lacZ* in respective polyphosphate genes. Wild-type F1C1 suspension was used as control where no color was appeared.

4.3.6 Expression study of polyphosphate metabolism homologues

4.3.6.1 lacZ expression assay

Expression study of *ppk1*, *ppk2*, *ppx* and *ppnk* polyphosphate metabolism homologues was done by measuring β-galactosidase enzyme (*lac*Z) activity in TRS1019, TRS1021, TRS1022 and TRS1023 strains. The assay in these insertion mutants were performed in two medium conditions viz. BG (nutrient rich) and Minimal Medium (nutrient poor) following Miller method (1972) [12].

Freshly streaked colonies of TRS1019, TRS1021, TRS1022 and TRS1023 strains were inoculated in BG media supplemented with ampicillin and gentamycin antibiotic and incubated for 24 hr at 28°C with shaking at 200 rpm. Fully grown bacterial cultures were independently pelleted down by centrifugation at 5000 rpm for 5 min in a centrifuge machine (Centrifuge 5424 R, Eppendorf, Germany), washed with sterile distilled water and resuspended in BG as well as in Minimal Medium. For all the strains, 1% of bacterial resuspension from each medium was transferred to respective BG and Minimal medium (10 ml each) and incubated overnight at 28°C with shaking at 200 rpm. After incubation, cultures were transferred to ice and

allowed to cool for 20 min to stop their growth. 2.0 ml of bacterial culture from each medium was pelleted down by centrifugation at 4°C and 13,000 rpm for 2 min. Pellets were then resuspended in 750 µl of Z-buffer (stored at 4°C) and mixed well by pipetting and vortexing. This step was repeated once to make the final volume 1.5 ml. Optical density of these suspensions was measured at 600 nm wavelength, considering Z-buffer as blank. In subsequent step, 750 µl of bacterial suspension was transferred to a 1.5 ml microfuge tube and 100 µl of chloroform followed by 50 µl of 0.1 % SDS was added to permeabilize the cells. The mixture was vortexed for 10 sec and incubated for 10 min at 28ºC. Following this, 150 µl of ONPG was added to each tube, mixed well and incubated at 37°C in a water bath (RCB20-Plus, Hoefer, USA). Time of ONPG addition was recorded. Once appearance of yellow color was observed, the reaction was terminated by adding 375 µl of 1 M Na_2CO_3 to each tube. Time of addition of Na_2CO_3 was recorded and then tubes were transferred to ice. Finally, the tubes were centrifuged for 10 min at 13,000 rpm to remove cell-debris. Optical densities of supernatants were measured at 420 nm and 550 nm, respectively and were recorded.

The enzyme activities were calculated in Miller Units by using the following equation:

Miller Units=1000x [(OD420-1.75 x OD550)]/ (T x V x OD600)

The unit gave the change in A420/min/ml of cells/OD600.

4.3.6.2 Real time expression assay

We also studied the real time expression of PolyP homologues in BG and MM (Minimal medium) using qRT-PCR approach.

RNA isolation and cDNA synthesis: *R. solanacearum* F1C1 was grown in BG and MM media at 28°C and cells were harvested after 18 hr of incubation. RNA was isolated using Trizol reagent (Ambion, Life Technologies) following manufacturer's instructions. RNA purity and integrity appropriate for downstream qRT-PCR applications were confirmed by measuring the ratio of absorbance at 260 nm/280 nm and 260 nm/230 nm in a BioSpectrometer (Eppendorf, Germany) and by visualization on a 1.0 % agarose gel. Moreover, DNA contamination was checked by performing PCR amplification of *R. solanacearum* specific gene. First-strand cDNA synthesis

was carried from 1.0 µg of purified RNA using the Maxima first strand cDNA synthesis kit performed according to the manufacturer's manual.

qRT- PCR reaction: Real time PCR reaction was performed using Maxima SYBR Green/ROX qPCR Master Mix as per manufacturer's instructions using *tuf* [13] as the reference gene. The cDNA was diluted in the ratio of 1:5 and used for the assay. Cycling was commenced with an initiation at 95 °C for 10 min, followed by 40 cycles of 15 sec at 95 °C and 30 sec at 57 °C and extension at 72°C for 30 sec. All qRT-PCR assays were carried out on the Mastercycler *ep realplex* Real Timer PCR system using two technical replication and non-template control. We kept similar reactions for all the genes examined; primers have been listed in Table 4.3. For each gene the threshold cycles (C_T) was an average of duplicate reactions, and the values were normalized with C_T of the *tuf*. Gene expression was calculated using the comparative $2-\Delta\Delta Ct$ method.

Table 4.3: List of primers used in the expression study of PolyP homologues

Sl. No	Name of the primer	Sequence	Remark
1	oRT11	5'- CATTCGTTGGAAACCACCAT-3'	*ppk1*
2	oRT12	5'- ATGAACTTCAAGCGCTCCAT-3'	
3	oRT05	5'- ACGACCATCGTCAAATGCTT-3'	*ppk2*
4	oRT06	5'- GGATCGAATTTCCAGTGCTT-3'	
5	oRT09	5'- GTCACTTCTTCCCGAATGGC -3'	*ppx*
6	oRT10	5'- TTGTCGTTGAAGCCGTTGAG -3'	
7	oRT13	5'- AGCGTCAACTTCGACATGC-3'	*ppnk*
8	oRT14	5'-TAGTTGTAGCCGACCGGATG -3'	
9	oRT45	5'- CGTCAAGAACATGATCACCG -3'	*tuf*
10	oRT46	5'- TGTCGCACTTGTTCAGGAAG-3'	

4.3.7 Creation of a deletion mutation in *ppk1* homologue of *R. solanacearum* F1C1

Deletion mutation in *ppk1* homologue of F1C1 was created by Ω cassette [14] insertion into the gene. The schematic representation of the steps followed has been shown in Fig.4.20. We designed the primers taking first 1- 2100 nucleotide sequences from F1C1 genome using primer -BLAST-tool (NCBI). The primer pair oFppk1.A and oRppk1.A (Table 4.2) was designed to amplify a 2054 bp size DNA fragment with a single *EcoRI* restriction site inside the amplified product (Fig.4.21). We used the PCR reaction recipe as described in section 4.3.5 and cycling condition as: (step1) initial denaturation at 95°C for 5 min; (step 2 for 35 cycles) denaturation at 95°C for 30 sec; annealing at 63.8°C for 30 sec; extension at 72°C for 2 min and (step3) final extension at 72°C for 7 min. The amplified product was visualized in 0.8% agarose gel (1xTAE, 70V, 200 mA & 35 min) and observed in a gel-doc system (E-gel imager, Thermo-Fisher Scientific, Mumbai, India). Subsequently, amplified DNA products were gel extracted using Qiagen gel extraction kit and confirmed by sequencing at Molbiogen (Guwahati, India). Subsequently, the amplicon was ligated to pGEM-T cloning vector (Promega, New Delhi, India) at 4°C for 16 hr and transformed into competent *E. coli* DH5α cells. Transformants were selected on LB-agar plate with ampicillin antibiotic added to it. Plasmid was isolated from positive transformant using Qiagen plasmid isolation kit and subjected to single digestion with restriction enzyme *EcoRI* and double digestion with *NdeI* and *NcoI* restriction enzymes for confirmation (Fig.4.22). After confirmation, recombinant plasmid pTP003 was linearized by digesting with *EcoRI* and ligated to ΩSpc cassette (2.0 kb size), digested with same enzyme to get the *ppk*:: ΩSpc::pGEM-T construct following standard protocol. The construct was then transformed into *E. coli* cells and transformants were selected on ampicillin and spectinomycin plates. From ampicillin and spectinomycin resistant colonies appeared on the plate, recombinant plasmid, pTP004 was isolated, checked by PCR to find out the insertion in the chromosomal copy of the respective gene (Fig.4.25) and further confirmed by restriction digestion (Fig.4.23).

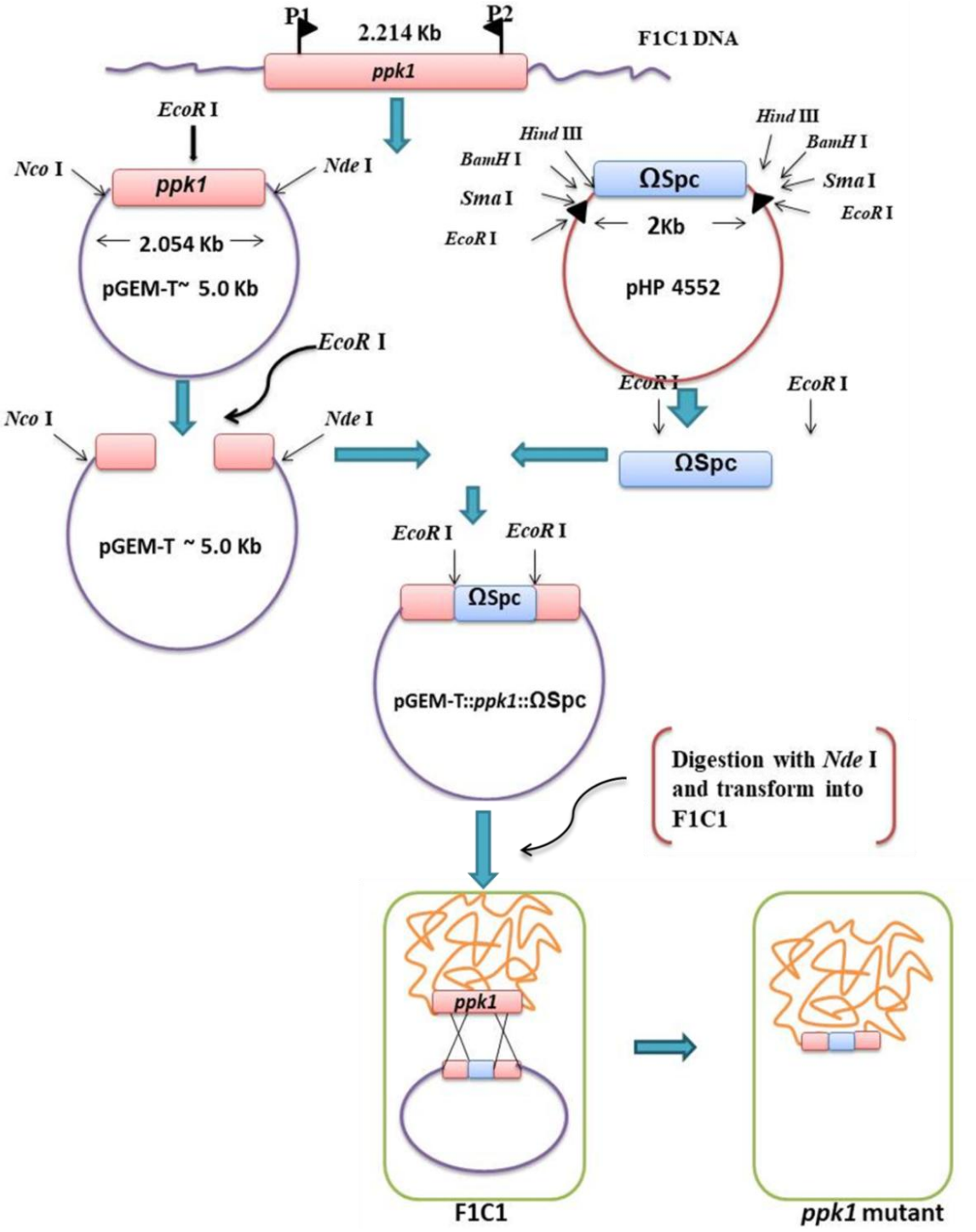


Fig.4.20: Schematic representation of Ω cassette fusion strategy by double homologous recombination event used to construct insertion/deletion mutagenesis.

In the next step, experiment was set for natural transformation in F1C1 with pTP004 construct to create deletion/insertion mutation in *ppk1* homolog of F1C1 following the protocol as described above. For that, pTP004 plasmid was linearized

with *NdeI* restriction enzyme present in pGEM-T vector, purified the construct and transformed into wild type F1C1. In transformed F1C1, occurrence of double-homologous recombination event leading to insertion of ΩSpc cassette and thereby substitution of a part of *ppk1* homologue created the deletion mutant of *ppk1*. The *ppk1* deletion mutants of F1C1 were selected on BG-agar plates containing spectinomycin antibiotic incubated at 28°C for 48 hr. Deletion event in mutants were confirmed by PCR with a forward primer (oFppk1.A) and a reverse primer designed at the 5' end of spectinomycin gene in ΩSpc cassette (oSpc5-01). An amplification of ~1.5 kb DNA band in mutant confirmed insertion of ΩSpc cassette which was absent in wild type F1C1 (Fig.4.25). One of the mutants, TRS1020 was studied further.

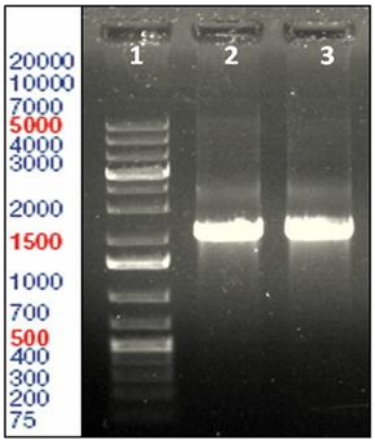


Fig.4.21: Agarose gel showing partial amplification of *ppk1* homologue in F1C1 genome. The ~2054 bp fragment amplified with oFppk1.A and oRppk1.A primers using F1C1 genomic DNA as template. Lane 1 has 1 kb plus DNA ladder. Lane 2 and 3 show ~2054 bp amplicon of *ppk1* homologue. The size of different DNA bands is showing at the side of the gel.

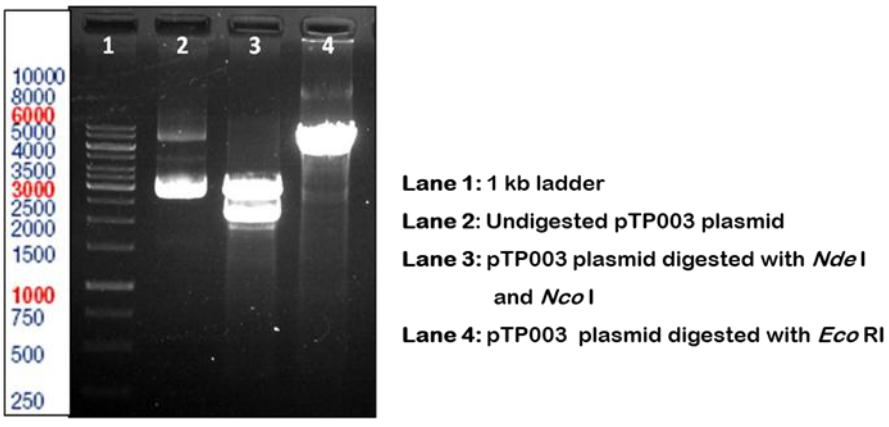


Fig.4.22: Agarose gel showing cloning confirmation of *ppk1* gene fragment in pGEM-T vector used for deletion mutation. Lane 1 has 1 kb DNA ladder. Lane 2 shows undigested pTP003 plasmid. Lane 3 shows double digestion of pTP003 plasmid with *NcoI* and *NdeI* enzymes yielding a ~3.0 kb and a ~ 2.0 kb size fragments. Lane 4 shows restriction digestion of pTP003 plasmid with *EcoRI*, yielding ~ 5.0 kb size linear construct. The size of different DNA bands is showing at the side of the gel.

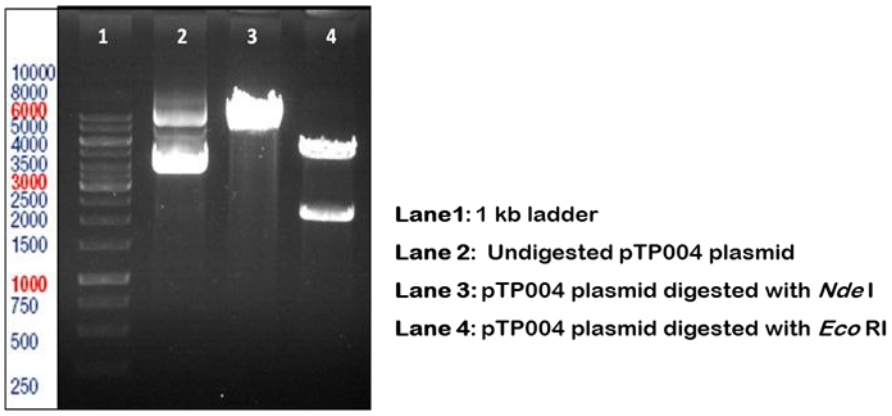


Fig.4.23: Agarose gel showing cloning confirmation of pTP004 recombinant harboring ΩSpc cassette. Lane 1 has 1 kb DNA ladder. Lane 2 shows undigested pTP004 vector construct. Lane 3 shows pTP004 digested with *NdeI* yielding a ~7.0 kb size linearized DNA band. Lane 4 shows digestion of pTP004 with *EcoRI* releases ΩSpc cassette (~2.0 kb size) and a ~ 5.0 kb size linear vector ligated with the *ppk1* gene fragment. The size of different DNA bands is showing at the side of the gel.

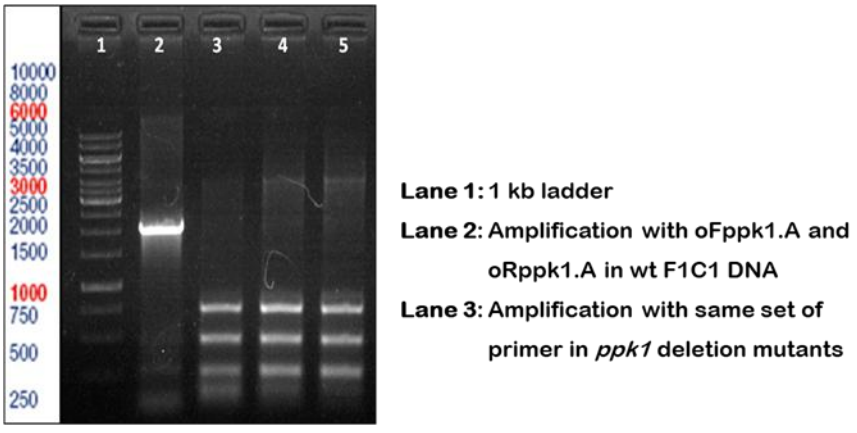


Fig.4.24: Agarose gel showing confirmation of deletion mutation in *ppk1* homologue of F1C1. Lane 1 has 1 kb DNA ladder. Lane 2 shows PCR amplification with primers oFppk1.A and oRppk1.A in wild type F1C1 yielding ~2054 bp DNA band. Lane 3 shows PCR amplification with same set of primers in *ppk1* deletion mutant that resulted no such band indicating disruption of gene sequence of *ppk1* homologue in that mutant. The size of different DNA bands is showing at the side of the gel.

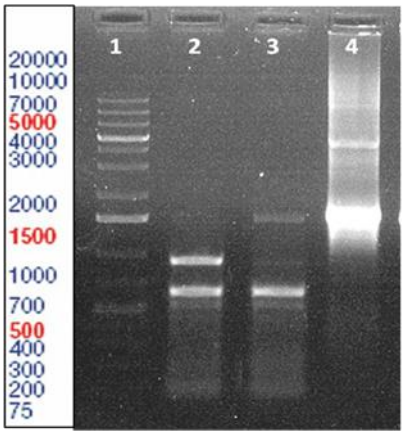
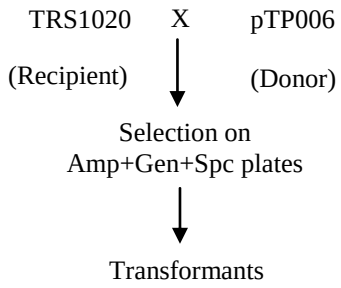


Fig.4.25: Agarose gel showing confirmation of deletion mutation in *ppk1* homologue of F1C1. Lane 1 has 1 kb plus DNA ladder. Lane 2 shows PCR amplification with primers oFppk1.A and oSpc5-01 in wild type F1C1 yielding no DNA band. Lane 3 shows PCR amplification with same set of primers in *ppk1* deletion mutant, TRS1020 that yielded a ~1500 bp DNA band indicating insertion of ΩSpc cassette in *ppk1* homologue leading to the creation of deletion mutant. Again, Lane 4 shows PCR amplification with same set of primers yielding a ~1500 DNA band in pTP004 plasmid, used to transform F1C1 to generate deletion mutant. The size of different DNA bands is showing at the side of the gel.

4.3.8 Creation of a *ppk1* and *ppk2* double mutant of *R. solanacearum* F1C1 strain

To create a double mutant strain of F1C1 carrying mutation in both *ppk1* and *ppk2* homologues, we followed the steps as:



We took *ppk1* deletion mutant strain, TRS1020 (harboring ΩSpc cassette in chromosomal copy of *ppk1*) as a recipient and made it competent by growing them in Minimal Medium supplemented with 10% glycerol. Donor plasmid pTP006 (*ppk2*::pCZ367) isolated from *E. coli* DH5α cells was then naturally transformed into competent TRS1020 cells following the methodology described in described earlier. Successful transformants carrying a deletion mutation in *ppk1* and an insertion mutation in *ppk2* were selected on BG-agar plates containing all Amp, Gen and Spc antibiotics together after 48 hr of incubation at 28°C. Double mutants obtained were

confirmed by performing PCR as done for confirmation of insertion mutation in *ppk2* and deletion mutation in *ppk1* as described in sections 4.3.5.2 & 4.3.7 respectively. TRS1024, double mutant strain was used in further studies.

4.3.9 Characterization of virulence phenotypes in polyphosphate metabolism mutants

4.3.9.1 Swimming motility assay

Media used for swimming motility was BG media (Bacto peptone, 10 g/l; Casamino acids, 1 g/l; Yeast extract, 1 g/l) supplemented with 0.2% agar. [15]. Swim plates were allowed to dry in a laminar air flow keeping blower on for ~ 1 hr. F1C1 and all polyphosphate mutants were freshly grown on BG agar plates and from the plates strains were point inoculated with a sterile toothpick onto the prepared swim plates. Motility was observed after 24 hr of incubation at 28º C.

4.3.9.2 Swarming motility assay

Media used for swarming motility assay consisted of BG medium with 0.7% agar [16]. Swarm plates were allowed to dry for ~ 2 hr at room temperature before being used. Bacterial strains were grown overnight in BG media incubated with shaking at 200 rpm and 28ºC until the OD_{600} of ~ 0.5. Cells were then harvested by centrifugation, washed twice with PBS and resuspended in sterile distilled water to an OD_{600} of ~ 0.2. For each strain, 1 µl of resuspension was spotted on swarm plates and incubated at 28º C for 3 days.

4.3.9.3 Twitching motility assay

Twitching motility in wild type F1C1 and polyphosphate mutants was studied following Ray et al., (2015) protocol [17]. Saturated culture of all the strains grown in BG media (without glucose) was diluted to 10^5 CFU/ml concentrations in sterile distilled water separately. 4 µl of each diluted inoculums were spot inoculated on BG-agar plates and incubated at 28°C. After 21 hr of incubation, plates were observed under transmitted light of an inverted fluorescence microscope (EVOS, Thermo-Fisher Scientific, Mumbai, India) equipped with 10X magnification.

4.3.9.4 Growth comparison of wild type and mutant strains

Cultures of wild type F1C1 and the mutant strains TRS1019, TRS1021, TRS1022 and TRS1023 were grown overnight and adjusted to an OD_{600} of 1.0. Then, 1% of each culture was inoculated into fresh BG broth and subsequently incubated at 28°C with 200 rpm shaking. The OD_{600} of each culture was monitored after every 2 hr interval. Three independent experiments were done in duplicate for each strain.

4.3.9.5 Hypersensitive Response (HR) assay

To check the hypersensitive responses of polyphosphate mutants in tobacco plants, freshly grown F1C1, TRS1019, TRS1021, TRS1022 and TRS1023 strains were cultured in BG medium supplemented with respective antibiotics for 24 hr at 28ºC and 200 rpm shaking incubator. Bacterial suspensions were prepared in sterile distilled water after harvesting the cells by centrifugation at 4000 rpm for 5-7 min. For all the strains, bacterial suspensions of saturated concentration (~10^9 CFU/ml) were used to infiltrate the tobacco leaves in the lower epidermis (ventral side) using needleless syringes. Tobacco leaves mock infiltrated in a similar way with sterile distilled water was kept as a control. The hypersensitive responses showed by the strains were observed after 24 hr as well as after 48 hr of infiltration.

4.3.9.6 Cellulase assay

Cellulase assay was performed in carboxymethyl cellulose plates (0.1% CMC; 1.5% agar) [18]. Wild type and mutant strains were grown on CMC for 48 hr at 28ºC. Next, plates were poured with 0.1 % (w/v) Congo red solution and kept at room temperature for 12 hr. Congo red solution was removed, washed with sterile water and then again flooded with 1 M NaCl (w/v) solution for 2 hr at room temperature. After the incubation, solution was discarded and plates were analyzed for formation of white zone suggesting cellulase activity.

4.3.9.7 Oxidative stress assay

Hydrogen peroxide was used to induce oxidative stress in cells. Wild type F1C1 and PolyP mutant strains were grown overnight and resuspended in sterile water by centrifugation. $OD_{600\ of}$ all strains were adjusted to 1.0 and 100 µl of each bacterial suspension was spreaded onto BG-agar plate supplemented with respective

antibiotics. After drying up of plates, 10 µl of a 250, 500, 1000, and 5000 mM hydrogen peroxide solution were added to the holes created on the plates. The diameter of inhibition zones were measured after 24 hr of incubation at 28ºC.

4.3.9.8 Virulence study in tomato seedlings

All the polyphosphate metabolism mutants were recruited for their virulence study in tomato seedlings. Inoculation was carried out by both root dip inoculation method [19] as well as by leaf clip inoculation method [20].

The root dip method of inoculation followed in brief was as: first, tomato seedlings were germinated on wet cotton and tissue paper bed in a plastic tray kept in a growth chamber (Orbitek, Scigenics Biotech, India) for a week. After 7 days of germination, seedlings were employed for the study. Next, we prepared bacterial inoculums of wild type F1C1 and all the polyphosphate metabolism mutants. For that, freshly grown cultures were pelleted down by centrifugation at 4000 rpm in a centrifuge machine for 10 min. Pellets were washed and resuspended in sterile distilled water to make final inoculums of ~10^9 CFU/ml. In the next step, from the germinated seedling tray, seedlings were picked and root of each seedling was then dipped in respective bacterial inoculums. Inoculated seedlings were transferred to sterile 1.5 ml microfuge tube, kept for a while for air exposure and 1 ml of sterile water was added to it. All the seedlings were inoculated in a similar way and incubated in a growth chamber (Orbitek, Scigenics Biotech, India).

The leaf clip method has already been described in chapter 2. A pair of sterile scissors dipped in the bacterial inoculums (10^9 CFU/ml) was used to clip off one-third of the cotyledon leaves in each 7 days old tomato seedling kept in microfuge tube. Both root dip and leaf clip inoculation was performed for all the strains separately in a set of 40 seedlings and was done in duplicates in a single experiment. Experiments were repeated three times. Seedlings mock inoculated with sterile distilled water were kept as control for both the methods. Inoculated seedling sets were then transferred to a growth chamber (Orbitek, Scigenics Biotech, India) maintained at 28°C, 12 hr photoperiod and 75% relative humidity. Disease in seedlings was analyzed and observations were recorded till 7^{th} day post inoculation. Data obtained were analyzed by Kaplan-Meier survival statistics and log-rank test [21].

4.3.10 Detection of PolyP accumulation in wild type F1C1 and PolyP mutants

We followed DAPI based approach to check the accumulation of PolyP molecules in wild type F1C1 and *ppk1, ppk2* and *ppx* insertion mutants [22]. DAPI fluorescence was measured in Fluorescence spectrometer at 550 nm wavelength excited at 415 nm (PERKIN-ELMER LS-55).

For this assay, we freshly grew the strains in BG media incubated for 24 hr. After the incubation, cells were harvested by centrifugation (5000 rpm, for 5 min) and washed twice with PBS buffer. Next, cell pellets were resuspended in PBS to an OD_{600} of ~ 1 and incubated with 2µM DAPI solution (in PBS) in room temperature for 2 hr. After the incubation cells were again resuspended in PBS buffer and scanned for DAPI fluorescence emmittance.

4.3.11 Creation of *ppk1* and *ppnk* complemented strain

For complementation study, we used pNP267 plasmid vector, a pRCG-GWY-based plasmid (Moterio et al. 2012) specifically designed for complementation study in *R. solanacearum* GMI1000 strain. The plasmid has two recombination sites designed for intergenic region of GMI1000. The homologous sequence of this non-coding region is also present in our F1C1 strain of *R. solanacearum*. Therefore, we used this vector pNP267 for stable chromosomal insertion of a full length functional copy of *ppk1* as well as *ppnk* homologue of F1C1 along with their promoter in mutant strains of *ppk1* and *ppnk* respectively. The map of complementation vector has been shown in Fig.4.26.

4.3.11.1 Confirmation of vector used for complemented study

The complementation vector was obtained from Dr. S. Genin's lab, LIPM, France and was transformed into *E. coli* DH5α strain. Successful transformants were selected on LB agar plate with gentamycin antibiotic. Subsequently, plasmid was isolated from transformed cells using Qiagen kit and double digested with *KpnI* and *HindIII* restriction enzymes to confirm the right vector that we had transformed as shown in Fig.4.27.

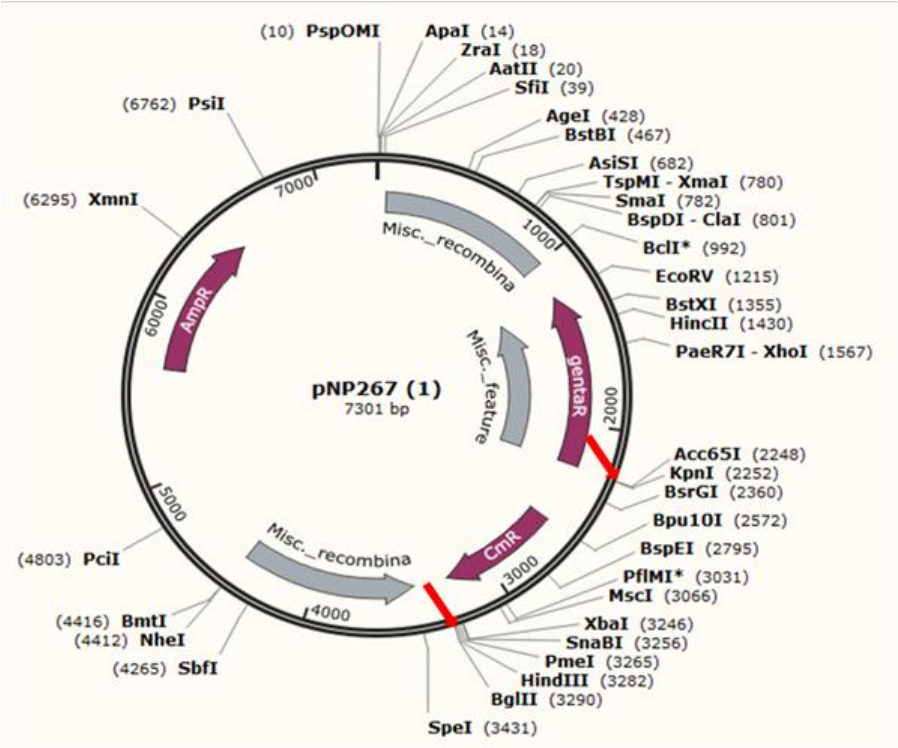


Fig.4.26: Map of pNP267 vector used for complementation study. The plasmid pNP267 has two recombination sites designed for an intergenic region of *R. solanacearum* genome. The *KpnI* and *HindIII* restriction sites marked with red arrow head were used to clone full length *ppk1* and *ppnk* homologue of F1C1.

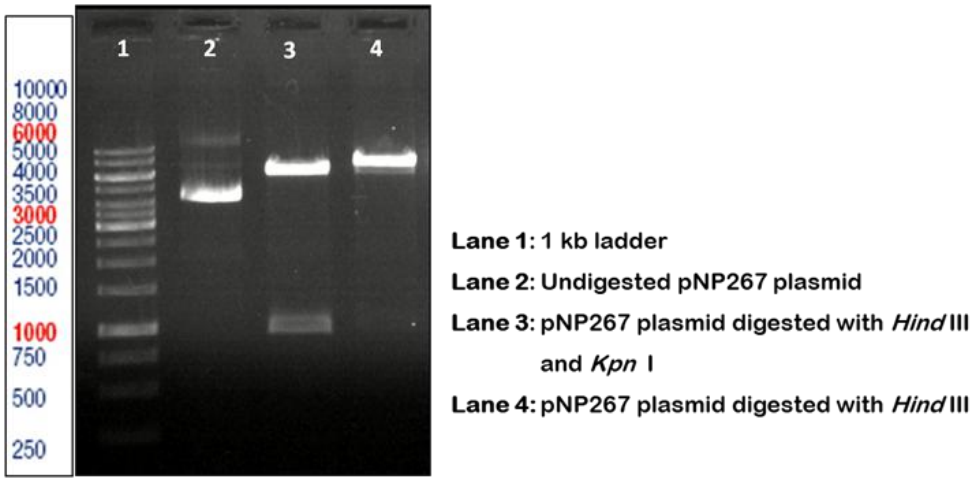


Fig.4.27: Agarose gel showing confirmation of pNP267 vector. Lane 1 has 1 kb DNA ladder. Lane 2 shows undigested pNP267 plasmid. Lane 3 shows double digestion of pNP267 plasmid with *HindIII* and *KpnI* enzymes releasing ~1.0 kb size DNA fragment and ~ 6.3 kb size linear vector. Lane 4 shows restriction digestion of pNP267 plasmid with *HindIII*, yielding ~ 7.3 kb size linear vector. The size of different DNA bands is showing at the side of the gel.

4.3.11.2 Cloning of full length ppk1 and ppnk homologues in pNP267 vector

For complementation study, we first took template sequences flanking full length *ppk1* and *ppnk* homologues along with their predicted promoters to design the primers using primer-BLAST tool. For both the genes, during primers designing *KpnI* and *HindIII* restriction sites were added at their 5'ends of forward and reverse primers respectively to clone into pNP267 vector using those sites. The *ppk1* and *ppnk* gene were amplified from wild type F1C1 using *Pfu* polymerase. Each PCR reaction was set in 20 µl reaction volume consisting of 2.0 µl of 10X Pfu buffer, 2 µl of 2 mM dNTP mix, 0.6 µl of DMSO, 0.5 µl of *Pfu* polymerase (2 U/µl), 1.0 µl of 5 µM forward primer and 1.0 µl of 5 µM reverse primer. Final volume was adjusted to 20 µl with Nuclease-free water. For amplification of *ppk1*, PCR conditions as well as annealing temperatures were standardized as: (step1) initial denaturation at 95°C for 3 min; (step 2 for 30 cycles) denaturation at 95°C for 30 sec; annealing at 63.3°C for 30 sec; extension at 72°C for 4 min and (step3) final extension at 72°C for 7 min.

The primer pair oFppk3 (5'-GCCGGTACCAAGCTGTTGGAACCCA TGTC-3') and oRppk3 (5'-GCCAAGCTTCTTTTCCGCCCTTCAAGC-3') successfully amplified ~ 2453 bp fragment as shown in Fig.4.28. Again for amplification of *ppnk*, we used similar reaction recipe as *ppk1*except annealing temperature of 56.5°C and the primer pair oFppnk3 (5'-GCCGGTACCC CGGAATACTTCGACAAGGT-3') and oFppnk3 (5'- GCCAAGCTTATCGG TTTTTGGCGTTACAG-3') successfully amplified ~ 1156 bp fragment (Fig.4.29).

The amplification was performed on a PCR machine (Eppendorf Mastercycler Nexus, Germany). Amplified products were visualized on 0.8% agarose gels (1xTAE, 70V, 200 mA & 35 min) and observed in a gel-doc system (E-gel imager, Thermo-Fisher Scientific, Mumbai, India). Subsequently, amplified DNA products were gel extracted using Qiagen gel extraction kit and sequenced at Molbiogen (Guwahati, India).

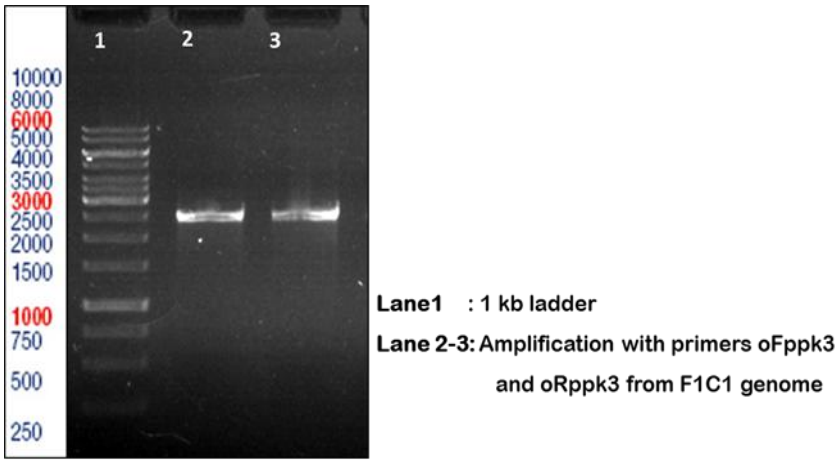


Fig.4.28: Agarose gel showing amplification of full length *ppk1* homologue along with promoter region in F1C1 genome. The ~ 2453 bp fragment amplified with oFppk3 and oRppk3 primers using F1C1 genomic DNA as template. Lane 1 has 1 kb DNA ladder. Lane 2 and 3 show ~2453 bp amplicon of *ppk1* homologue. The size of different DNA bands is showing at the side of the gel.

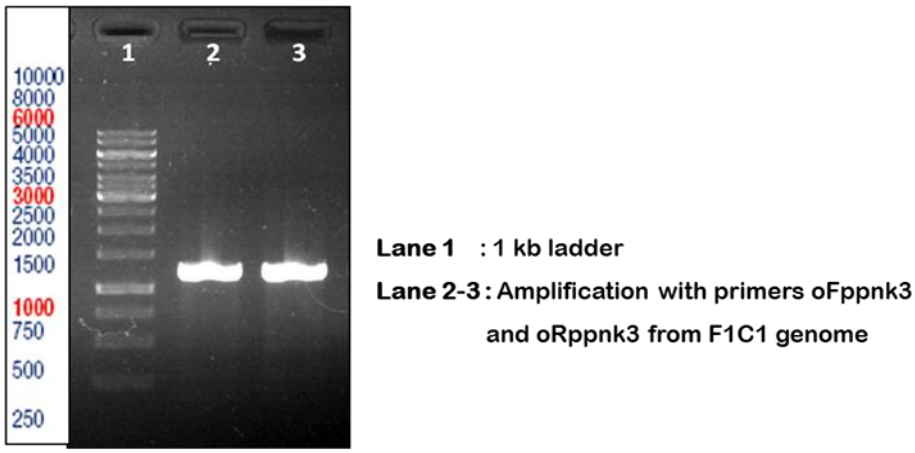


Fig.4.29: Agarose gel showing amplification of full length *ppnk* homologue along with promoter region in F1C1 genome. The ~ 1156 bp fragment amplified with oFppnk and oRppnk primers using F1C1 genomic DNA as template. Lane 1 has 1 kb DNA ladder. Lane 2 and 3 show ~1156 bp amplicon of *ppnk* homologue. The size of different DNA bands is showing at the side of the gel.

The amplified products of both the genes were then linearized with *KpnI* and *HindIII* enzymes and ligated separately to the pNP267 vectors digested with the same enzymes using standard protocol at 4ºC for 16 hr. Ligated constructs were then transformed into *E. coli* DH5α cells and positive transformants were selected on LB agar plates resistant against ampicillin and gentamycin but sensitive for chloramphenicol. Recombinant plasmids were then confirmed by PCR amplification and restriction digestion as shown in Fig.4.30 and Fig.4.31.

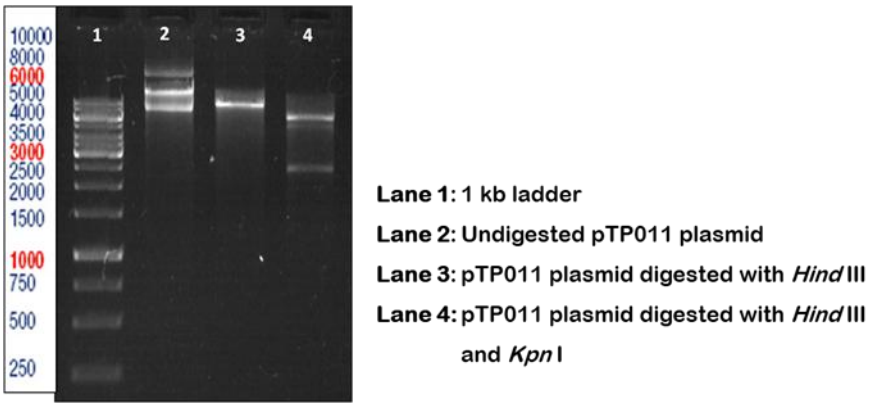


Fig.4.30: Agarose gel showing cloning confirmation of *ppk1* in pNP267 vector used for complementation. Lane 1 has 1 kb DNA ladder. Lane 2 shows undigested pTP011 plasmid. Lane 3 shows restriction digestion of pTP011 plasmid with *HindIII*, yielding ~ 8.7 kb size linear construct. Lane 4 shows double digestion of pTP011 plasmid with *HindIII* and *KpnI* enzymes yielding a ~ 6.3 kb vector and a ~ 2453 bp size insert. The size of different DNA bands is showing at the side of the gel.

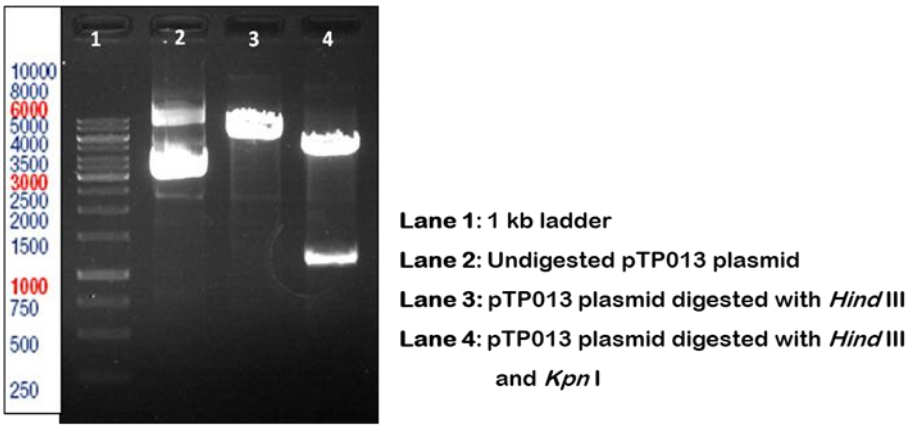


Fig.4.31: Agarose gel showing cloning confirmation of *ppnk* in pNP267 vector used for complementation study. Lane 1 has 1 kb DNA ladder. Lane 2 shows undigested pTP013 plasmid. Lane 3 shows restriction digestion of pTP013 plasmid with *HindIII*, yielding ~ 7.5 kb size linear construct. Lane 4 shows double digestion of pTP013 plasmid with *HindIII* and *KpnI* enzymes yielding a ~ 6.3 kb vector and a ~ 1156 bp size insert. The size of different DNA bands is showing at the side of the gel.

4.3.11.3 Insertion of Spectinomycin cassette (ΩSpc) into pNP267::ppk1 and pNP267::ppnk construct

Since, the complementation vector pNP267 as well as insertion mutants *ppk1*and *ppnk* were resistant to same antibiotics ampicillin and gentamycin, for selection of transformants, we modified both the construct pNP267::*ppk1*and pNP267::*ppnk* by inserting a Spectinomycin cassette (ΩSpc) using *HindIII* enzyme.

The resulting pNP267::*ppk1*::ΩSp construct was then transformed into *E. coli* and successful transformants were confirmed with PCR amplification and restriction digestion .

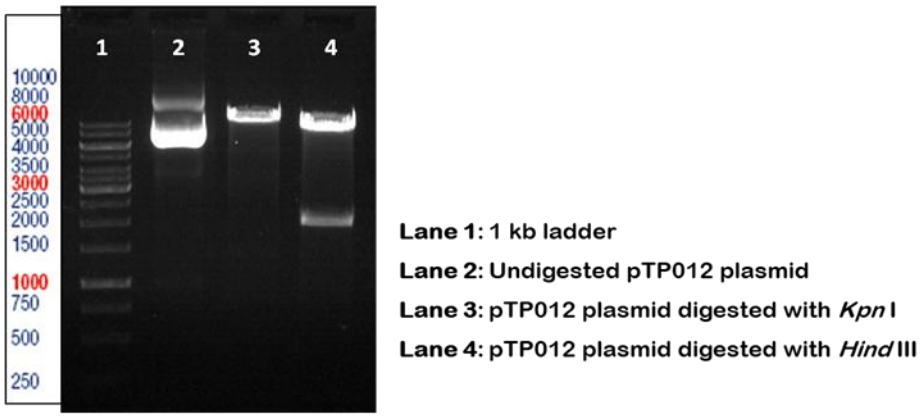


Fig.4.32: Agarose gel showing cloning confirmation of pTP012 plasmid. Lane 1 has 1 kb DNA ladder. Lane 2 shows undigested pTP012 vector construct. Lane 3 shows pTP012 digested with *KpnI* yielding a ~10.7 kb size linearized vector. Lane 4 shows digestion of pTP012 with *HindIII* releasing ΩSpc cassette (~2.0 kb size) & a ~ 8.7 kb size linear vector ligated with the *ppk1* gene fragment. The size of different DNA bands is showing at the side of the gel.

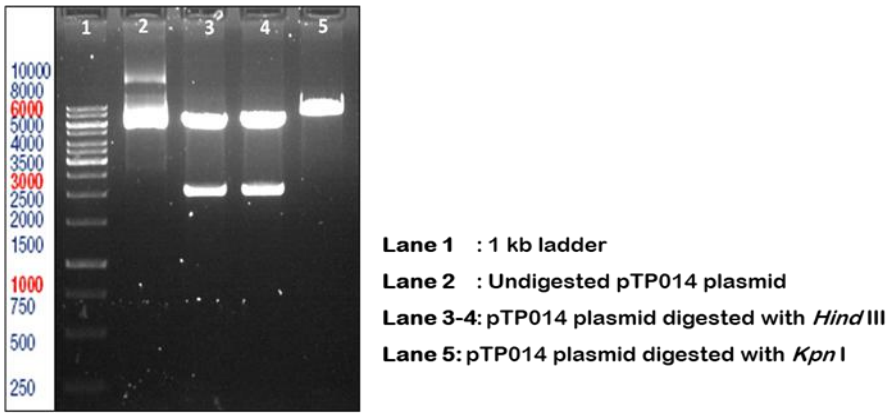


Fig.4.33: Agarose gel showing cloning confirmation of pTP014 plasmid. Lane 1 has 1 kb DNA ladder. Lane 2 shows undigested pTP014 vector construct. Lane 3 and 4 shows digestion of pTP014 with *HindIII* releasing ΩSpc cassette (~2.0 kb size) & ~ 7.5 kb size linear vector ligated with the *ppnk* gene. Lane 5 shows pTP014 digested with *KpnI* yielding a ~ 9.5 kb size linearized vector. The size of different DNA bands is showing at the side of the gel.

4.3.11.4 Transformation of pTP012 and pTP014 into ppk1 and ppnk insertion mutant strains of F1C1

After getting pTP012 and pTP014 plasmids, next step was to transform these plasmids into *ppk1* and *ppnk* insertion mutant background. For natural transformation, TRS1019 (*ppk1*::pCZ367::F1C1) and TRS1023 (*ppnk*::pCZ367::F1C1) were made

competent by growing them in Minimal Medium supplemented with 10% glycerol and were transformed with recombinant plasmids pTP012 and pTP014. Successful transformants were selected on BG-agar plate containing ampicillin, gentamycin and spectinomycin antibiotics together after incubation of 48 hr at 28ºC. The *ppk1* and *ppnk* transformants obtained harboring full copy of *ppk1* and *ppnk* gene respectively by the double homologous recombination event were confirmed by PCR amplification. For PCR amplification, a primer pair (oF612, oR611) specific to a region of the complementation vector, pNP267 that was also inserted along with the targeted full length gene at its upstream region was used. The primer set oNP611 and oNP612 successfully amplified ~798 bp size DNA band in both complemented strains (Fig.4.34; Fig.4.35).

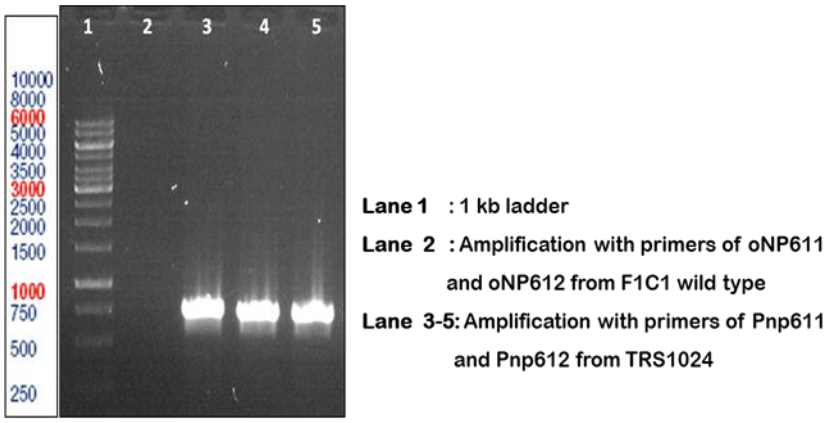


Fig.4.34: Agarose gel showing confirmation of double homologous recombination event in *ppk1* complemented strain. Lane 1 has 1 kb DNA ladder. Lane 2 shows PCR amplification with primers oNP611 and oNP612 in wild type F1C1 yielding no DNA band. Lane 3, 4 and 5 shows PCR amplification with same set of primers in *ppk1* complemented strain resulting ~ 798 bp size DNA fragment. The size of different DNA bands is showing at the side of the gel.

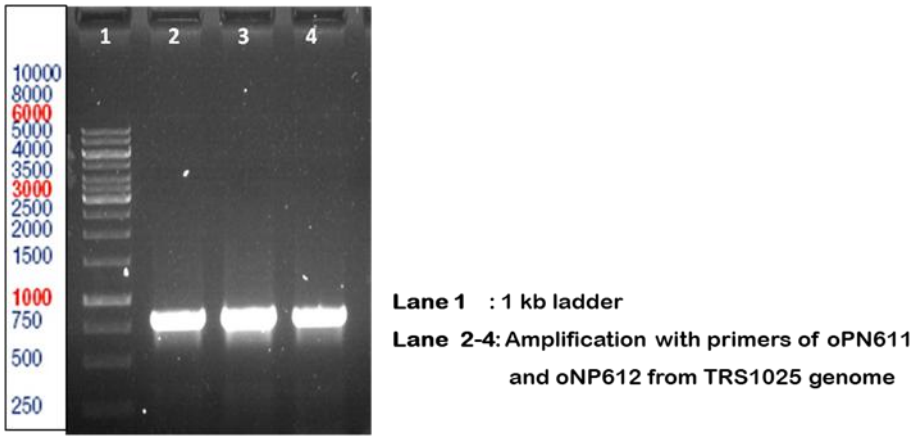


Fig.4.35: Agarose gel showing confirmation of double homologous recombination event in *ppnk* complemented strain. Lane 1 has 1 kb DNA ladder. Lane 2, 3 and 4 shows PCR amplification with primers oNP611 and oNP612 in *ppnk* complemented strain resulting ~ 798 bp size DNA fragment. The size of different DNA bands is showing at the side of the gel.

Moreover, amplification of full length *ppk1* gene with primer pair oFppk3, oRppk3 and full length *ppnk* gene with primer pair oFppnk3 and oRppnk3 indicated chromosomal insertion of respective full copy of these genes leading to the creation of complemented strains (Fig.4.36; Fig.4.37). The *ppk1* complemented strain TRS1024 and strain *ppnk* complemented strain TRS1025 were used for further studies.

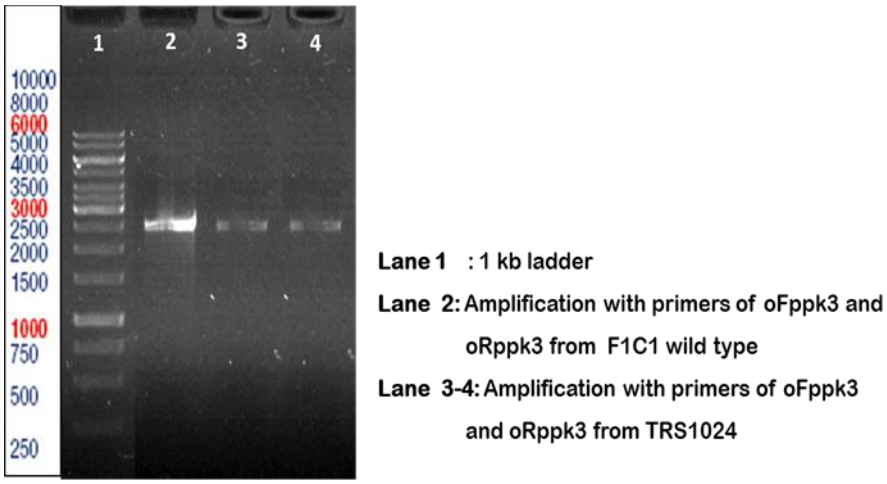


Fig.4.36: Agarose gel showing confirmation of full length *ppk1* insertion in TRS1024 genome. The ~ 2453 bp fragment amplified with oFppk3 and oRppk3 primers using TRS1024 genomic DNA as template. Lane 1 has 1 kb DNA ladder. Lane 2 shows ~2453 bp amplicon of *ppk1* homologue in wild type F1C1. Lane 3 and 4 show same size of amplicon with same set of primer in TRS1024. The size of different DNA bands is showing at the side of the gel.

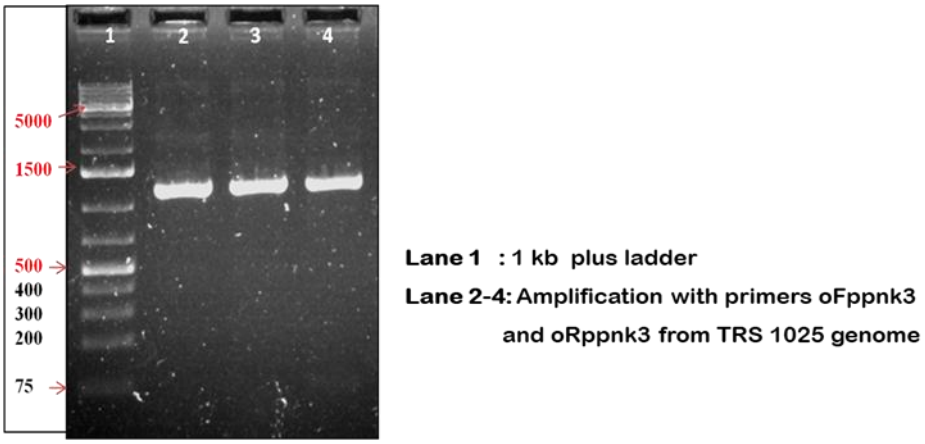


Fig.4.37: Agarose gel showing confirmation of full length *ppnk* insertion in TRS1025 genome. The ~ 1156 bp fragment amplified with oFppnk3 and oRppnk3 primers using

TRS1025 genomic DNA as template. Lane 1 has 1 kb plus DNA ladder. Lane 2, 3 and 4 shows ~1156 bp amplicon of *ppnk* homologue. The size of different DNA bands is showing at the side of the gel.

4.4 Results:

4.4.1 Polyphosphate metabolism genes are conserved in *R. solanacearum* F1C1

The sequences of polyphosphate metabolism genes *ppk1, ppk2, ppx* and *ppnk* were identified in F1C1 sequenced genome considering *R. solanacearum* GMI1000 as reference strain. These sequences were independently subjected to homology search (BlastN, NCBI) and significant homology to corresponding genes in already sequenced genomes of different strains could be observed as shown in Table 4.4. This observation confirmed the conservation of all these genes in F1C1 strain.

Table 4.4: Conservation of polyphosphate gene homologues in *R. solanacearum* F1C1

Sl No	*R. solanacearum* strains	Phylotype	*ppk1* (2214 bp) % identity	*ppk2* (828 bp) % identity	*ppx* (1584bp) % identity	*ppnk* (930 bp) % identity
1	F1C1	I	100	100	100	100
2	GMI1000	I	99	99	99	99
3	FQY_4	I	99	99	99	99
4	YC45	I	99	99	99	99
5	Po82	IIB	95	93	94	96
6	CFBP2957	IIA	94	84	94	95
7	CMR15	III	98	98	98	99
8	PSI07	IV	96	95	95	97

4.4.2 Creation of *ppk1, ppk2, ppx* and *ppnk* insertion mutants

The *ppk1, ppk2, ppx* and *ppnk* mutants of F1C1 were created by *lacZ* reporter gene fusion as described in method section. The pCZ367 vector possesses a *lacZ* gene; insertion of which causes disruption of target gene function and allows *lacZ* expression under the regulation of that gene promoter. The sites of *lacZ* gene insertion in targeted genes are shown below.

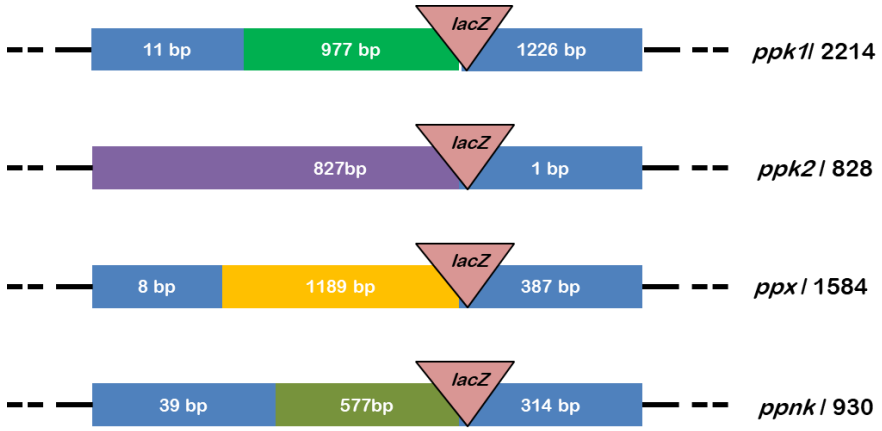


Fig.4.38: Schematic representation of mutation sites in polyphosphate metabolism homologues. Insertion of *lacZ* in different positions of corresponding genes created the mutant strains of F1C1.

4.4.3 Creation of *ppk1* deletion mutant

We have also generated a deletion mutant of *ppk1* homologue to ensure that the phenotype attributed by *ppk1* insertion mutant is more conclusive. We used ΩSpc cassette for creation of deletion/insertion mutation. Double homologous recombination event allowed the deletion of a fragment of *ppk1* homologue substituted by insertion of ΩSpc cassette into the gene. Mutants obtained were confirmed by PCR as described in method section.

Fig.4.39: Schematic representation of insertion/deletion mutation site in *ppk1* homologue. Insertion of **Ω**Spc cassette in the *EcoRI* restriction site of the gene substituted a part of *ppk1* that created a deletion mutant strain of F1C1.

4.4.4 Creation of *ppk1* and *ppk2* double mutant

Since *R. solanacearum* F1C1 possesses two homologues encoding two forms of polyphosphate kinase enzymes, we were interested to create a double mutant of *ppk1* and *ppk2* to check whether there will be any synergistic effect on mutant phenotypes. Steps have been discussed in method section. One double mutant strain, TRS1024 was used in virulence studies.

4.4.5 Polyphosphate metabolism genes showed higher expression in minimal medium

We checked the expression of PolyP metabolism genes in mutant background i.e. in TRS1019 (*ppk1*), TRS1021 (*ppk2*), TRS1022 (*ppx*) and TRS1023 (*ppnk*) strains by assessing β-galactosidase activity of *lac*Z genes expressed under the respective gene promoters. We have used nutrient rich BG medium and nutrient poor Minimal Medium conditions for the study. In Minimal Medium, expression of all polyphosphate genes was found to be higher in comparison to BG medium. In case of *ppk1*, expression was around five fold more while it was around four fold more in case of *ppnk.* In case of *ppk2* and *ppx*, the fold difference was lesser; around 2 fold for both the genes. Higher expression in Minimal Medium which mimic nutrient limiting plant environment gave an indirect indication of their expression *in planta* during pathogenesis.

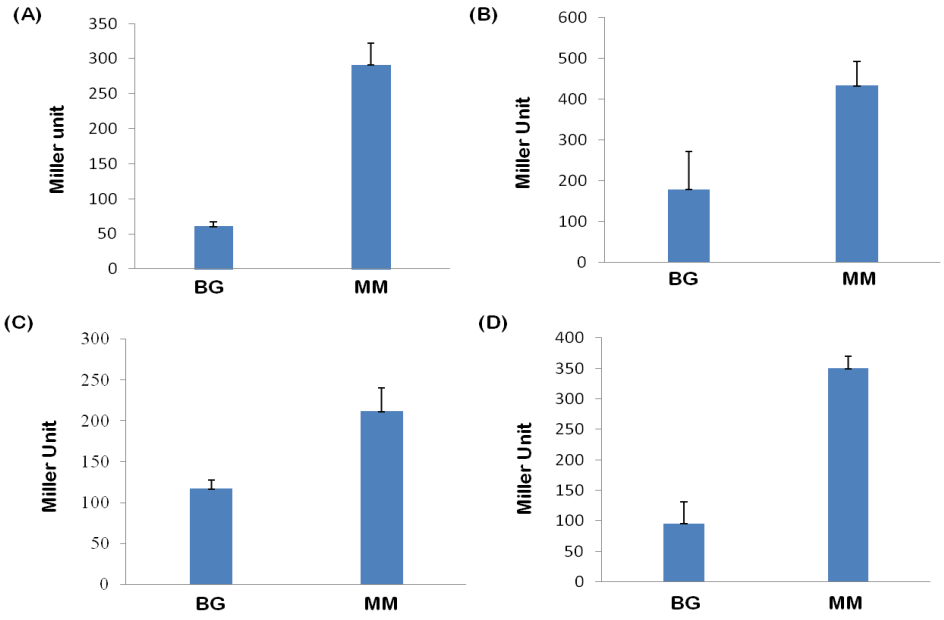


Fig.4.40: Expression pattern of polyphosphate metabolism genes. Expression was studied in BG and Minimal Medium of (A) *ppk1* (B) *ppk2* (C) *ppx* and (D) *ppnk*. The Y-axis represents β-galactosidase activity expressed in Miller units and the X-axis represents the medium conditions. Standard errors were calculated from standard deviations independently.

We further checked the expression of PolyP genes by qRT-PCR in wild type F1C1. The real time expression pattern of the genes *ppk1*, *ppk2* and *ppnk* exhibited

higher expression in MM medium compared to BG medium. However, *ppx* expression did not correlate with *lac*Z expression pattern.

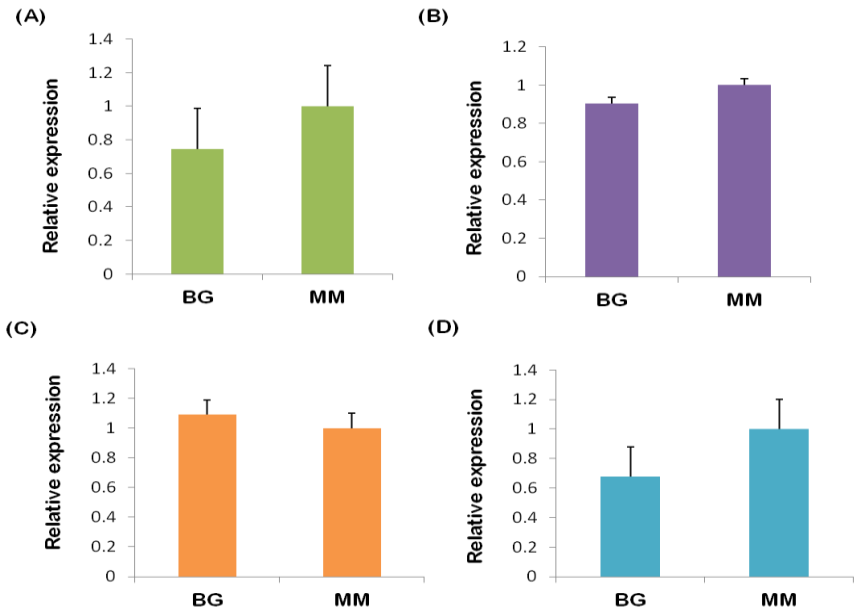


Fig.4.41: Expression pattern of polyphosphate metabolism genes by qRT-PCR. Expression was studied in BG and Minimal Medium of (A) *ppk1* (B) *ppk2* (C) *ppx* and (D) *ppnk*. The X-axis represents media conditions and the Y-axis represents relative expression. Standard errors were calculated from standard deviations independently.

4.4.6 *ppk1, ppk2, ppnk* mutants were swimming motility deficient

Swimming motility test for *R. solanacearum* strains was done in soft agar plates. Swimming proficient bacteria grows radially in all directions and distinct white zone can be observed. We observed distinct radial zone covering the full plate in case of wild type F1C1 after 24 hr of incubation. Interestingly, we found *ppk1* and *ppnk* mutants to be highly reduced for swimming motility compared to wild type. We observed swimming motility deficiency in *ppk2* mutant also while *ppx* mutant was observed to be proficient for this motility. We also recruited *ppk1* and *ppk2* double mutant for this study which we found to be swimming motility deficient similar to *ppk1* insertion/deletion mutant, the strain in background of which it was created (Fig.4.42). These results suggested the involvement of PolyP molecule in the swimming motility of F1C1.

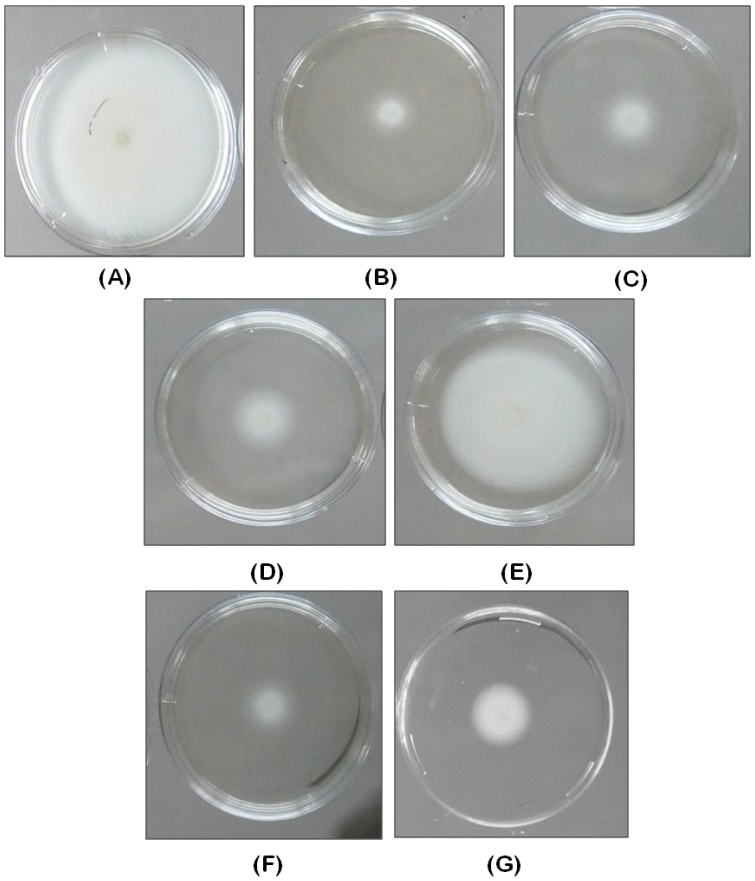


Fig.4.42: Swimming motility of polyphosphate metabolism mutants. Swimming motility was assayed on soft agar plate**s** of (A) wild type F1C1 (B) *ppk1* insertion mutant (C) *ppk1* insertion/ deletion mutant (D) *ppk2* (E) *ppx* (F) *ppnk* (G) *ppk1::ppk2* double mutant. F1C1 showed swimming proficiency covering the whole plate after 24 hr of incubation. Both the mutants of *ppk1* and *ppnk* were swimming motility deficient while *ppx* mutant was proficient for this motility almost like wild type. The *ppk2* mutant also showed reduced swimming motility but more than *ppk1* or *ppnk* mutant. The double mutant of *ppk1* and *ppk2* showed motility deficiency similar to *ppk1* insertion/ deletion mutant.

4.4.7 The *ppk1* mutant showed reduced swarming motility

The swarming motility was studied in BG medium added with 0.7% agar. After 3 days of incubation at 28°C, we observed irregular branching like colony morphology at the periphery indicated swarming motility in wild type *R. solanacearum* F1C1. We observed reduced swarming motility in case of *ppk1* mutant while *ppx* mutant showed similar pattern like in wild type. However, we could not accurately attribute swarming motility phenotype to *ppk2* and *ppnk* mutants. These assay indicated the role of PPK in flagellar dependent swarming motility of F1C1.

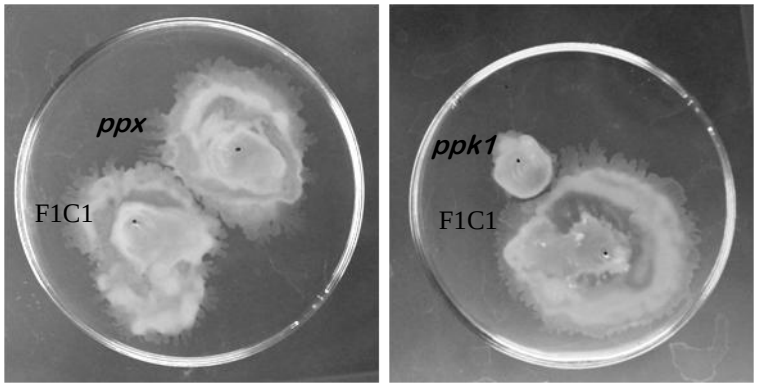


Fig.4.43: Swarming motility of polyphosphate metabolism mutants. Swarming motility of (A) *ppk1* insertion mutant and (B) *ppx* was assayed on BG agar plate with 0.7% agar. The *ppk1* mutant showed swarming deficiency while *ppx* mutant was swarming proficient like the wild type F1C1.

4.4.8 Polyphosphate mutants are twitching motility proficient

R. solanacearum has the ability to migrate on or adhere to solid surfaces that is mediated by Type IV-pilus system. This ability termed twitching motility in *R. solanacearum* has been reported to be an important virulence factor [23,24]. We checked all the polyphosphate mutants generated for their twitching motility on plates. BG plates spotted with strains were observed after ~ 21 hr of incubation. Finger like protrusions emerging out of the spot edges were observed on plates of wild type as well as mutants, suggesting all the polyphosphate mutants were twitching motility proficient (Fig.4.44).

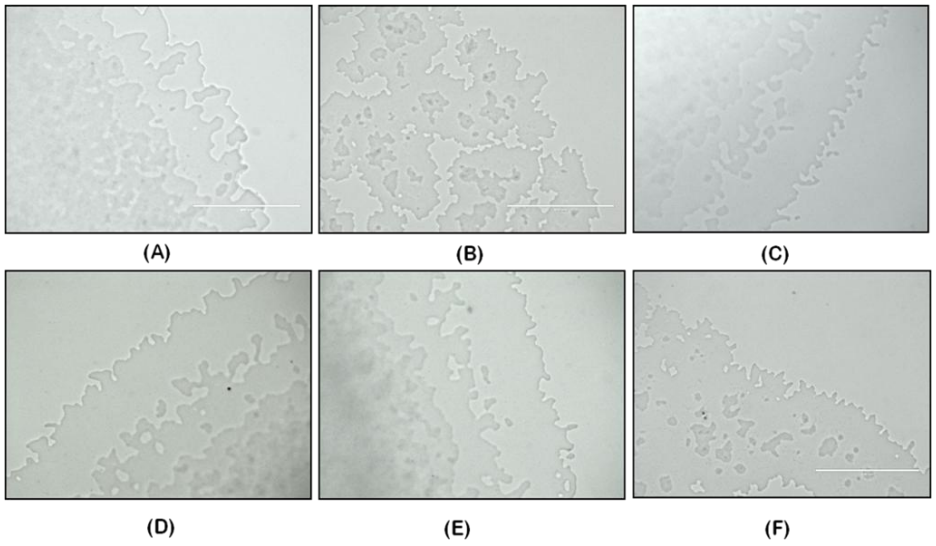


Fig.4.44: Twitching motility on BG agar plate. Twitching motility of polyphosphate mutants were observed under microscope in 10X magnification after 21 hr of incubation. (A)

F1C1 (B) *ppk1* insertion mutant (C) *ppk1* insertion/deletion mutant (D) *ppk2* (E) *ppx* and (F) *ppnk* mutant. All the mutants showed twitching motility proficiency similar to wild type F1C1.

4.4.9 Polyphosphate metabolism mutants were growth proficient like the wild type

To check whether the swimming motility deficiency observed in case of *ppk1*, *ppnk* and *ppk2* mutants were due to growth deficiency of the bacteria, we compared their growth rate with wild type F1C1. We determined the growth curve for all the mutant strains TRS1019, TRS1021, TRS1022 and TRS1023 and found to grow like the wild type *R. solanacearum* F1C1. This finding suggested that the deficiency observed in case of mutants were not related with their growth in culture.

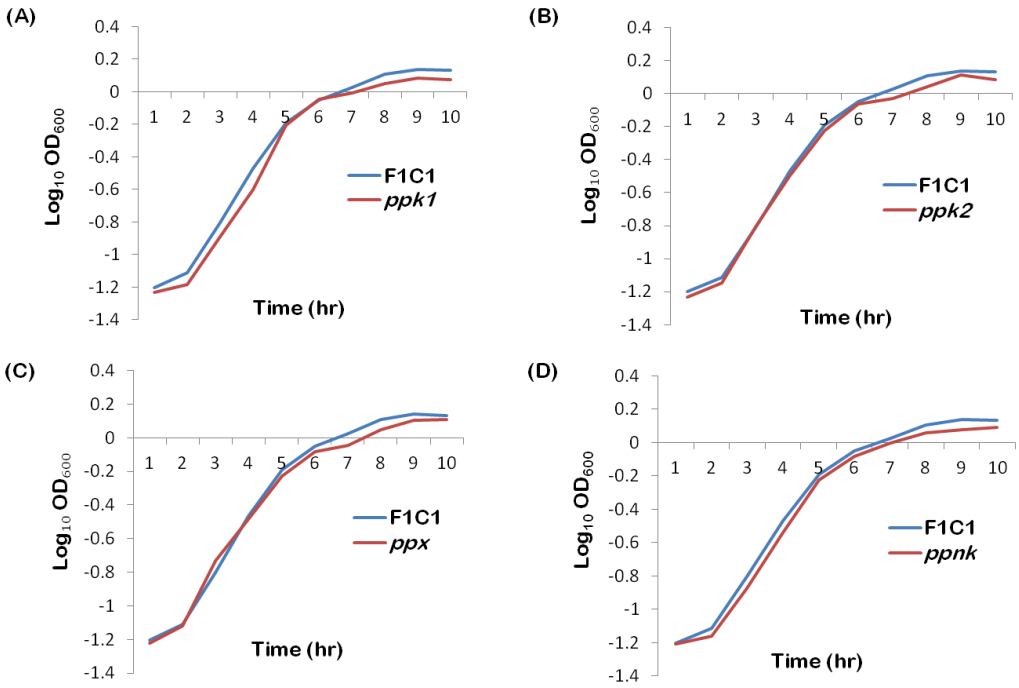


Fig.4.45: Growth curve of wild type and PolyP mutant strains. Comparative growth curves of F1C1 with (A) *ppk1* (B) *ppk2* (C) *ppx* and (D) *ppnk* mutant.

4.4.10 Polyphosphate metabolism mutants were proficient for Hypersensitive Response (HR)

Hypersensitive response is a feature of plant disease resistance which is a localized cell death response that prevents the spread and establishment of the pathogen in host. It depends on classical gene-for-gene interaction between pathogen

and the host. We checked all the PolyP metabolism mutants for their response towards tobacco plant host. Infiltration of tobacco leaves with bacterial suspensions of all the strains (saturated concentration) resulted elicitation of HR response like the wild type *R. solanacearum* F1C1 strain suggesting no influence of polyphosphate molecules upon T3SS (Fig.4.46).

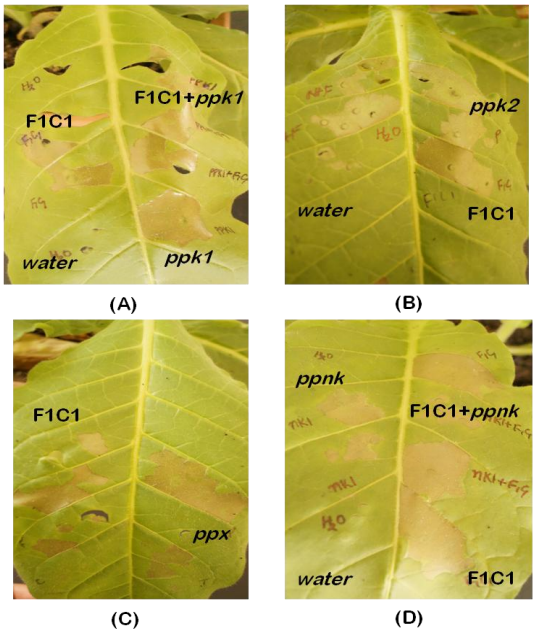


Fig.4.46: HR assay of polyphosphate metabolism mutants. Tobacco leaves were infiltrated with (A) *ppk1* (B) *ppk2* (C) *ppx* and (D) *ppnk* mutants. All the PolyP mutants were proficient for eliciting hypersensitive response in tobacco plant after 48 hr of infiltration similar to the wild type F1C1.

4.4.11 Polyphosphate metabolism mutants were cellulase proficient

To degrade the host cell wall, *R. solanacearum* secretes cellulase enzyme through type II protein secretion system (T2SS) and plays important role in virulence [25]. We tested all the PolyP mutants for their extracellular cellulase activity in CMC-agar plate. We found that the activity of cellulase enzymes of TRS1019, TRS1021, TRS1022 and TRS1023 were un-affected as white halo were distinctly observed like in case of wild type F1C1. This suggested that PolyP metabolism functions have no role in connection with T2SS in *R. solanacearum*.

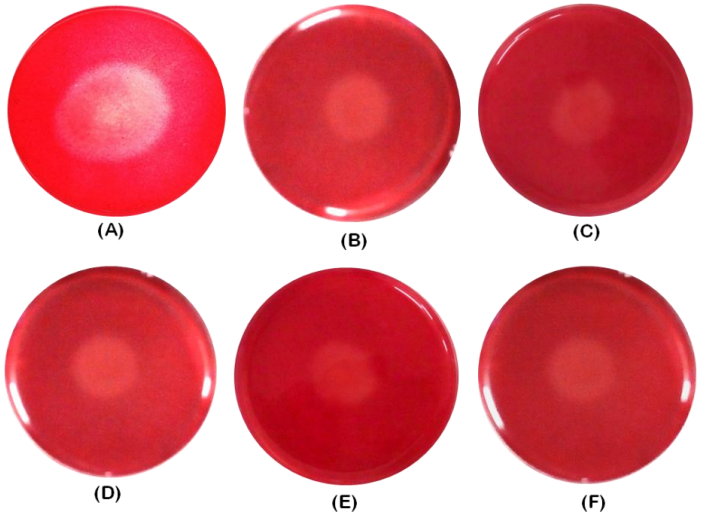


Fig.4.47: Cellulase assay on CMC agar plates. (A) F1C1 (B) *ppk1* insertion mutant (C) *ppk1* insertion/deletion mutant (D) *ppk2* (E) *ppx* and (F) *ppnk* mutants on CMC agar plates. White-halo in the plates indicated that all PolyP mutants were proficient for extracellular cellulase secretion.

4.4.12 PolyP mutants exhibited tolerance to oxidative stress

To determine whether PolyP metabolism genes have any role in oxidative stress tolerance in *R. solanacearum* F1C1, we treated wild type and mutant cells with different concentrations of H_2O_2 in plates and resulting inhibition zones were measured. We could not find out any significant differences between wild type and mutant strains indicating the tolerance of PolyP mutants to high dose of H_2O_2 upto 5000 mM like in wild type. *R. solanacearum* PolyP might not have strong role in oxidative stress tolerance to hydrogen peroxide in culture.

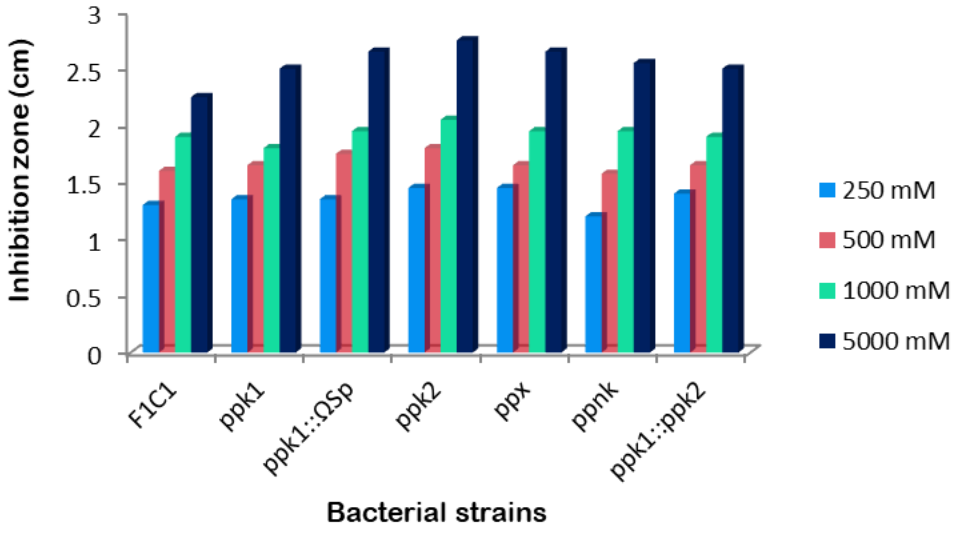


Fig.4.48: **Determination of hydrogen peroxide sensitivity of PolyP mutant strains.** Wild type F1C1 and PolyP mutants were exposed to 250 mM, 500 mM, 1000 mM and 5000 mM concentrations of hydrogen peroxide on plate. The zone of inhibition is presented in centimeter and was analyzed from two independent experiments.

4.4.13 *ppk1* and *ppnk* mutants were virulence deficient in tomato seedlings

To check the role of the targeted polyphosphate metabolism genes in virulence of *R. solanacearum* F1C1 if any, we performed virulence assay on tomato host. Virulence assay was performed employing 6-7 days old tomato seedlings as described in the section 4.3.9.8. Inoculation of the *ppk1* insertion mutant (TRS1019), *ppk1* deletion mutant (TRS1020), *ppk2* insertion mutant (TRS1021), *ppx* insertion mutant (TRS1022), *ppnk* insertion mutant (TRS1023), and *ppk1* and *ppk2* double mutant (TRS1024) on tomato seedlings were done by the root inoculation method. Moreover, we checked virulence phenotypes of TRS1019, TRS1021, TRS1022 and TRS1023 strains by leaf clip inoculation method also.

We compared virulence property of each gene mutant with wild type F1C1. In case of double mutant, virulence was compared with *ppk1* insertion/ deletion mutant and *ppk2* in another experiment. Virulence data were recorded till 7 days post inoculations and statistical analysis was done by using Kaplan-Meier survival probability and log-rank test.

In the virulence assay of *ppk1* insertion mutant, we found this mutant to be drastically reduced for virulence compared to wild type in tomato seedlings by root inoculation method. F1C1 killed more number of seedlings which was very distinct from 3 DPI onwards. By the end of 7 DPI, 35% of the seedlings were killed in case of seedlings inoculated with *ppk1* mutant while in case of wild type F1C1; it was more than 75%. The deficiency in virulence was statistically very significant (Fig.4.49).

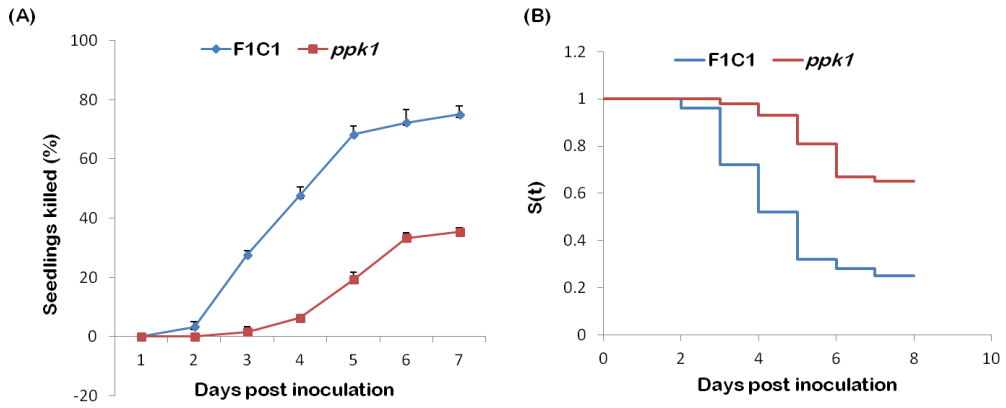


Fig.4.49: Virulence data analysis of *ppk1* insertion mutant by root inoculation. TRS1019 strain was inoculated in tomato seedlings by root dip method. (A) Line graph showing percentage of seedlings killed against days post inoculation (DPI). Each data point is an average of three independent experiments with two replicates. Error bars are depicting standard errors. (B) Kaplan–Meier survival curve depicting survival probability of infected seedlings. The *ppk1* insertion mutant exhibited significant virulence deficiency in compared to the wild type F1C1 ($P < 0.001$; log-rank test).

In leaf clip inoculation method also, we observed almost similar magnitude of virulence deficiency of *ppk1* mutant in compared to wild type (Fig.4.50). Though the disease appearance and progression was rapid in root inoculation method; number of seedlings killed was more in case of leaf clip inoculation method.

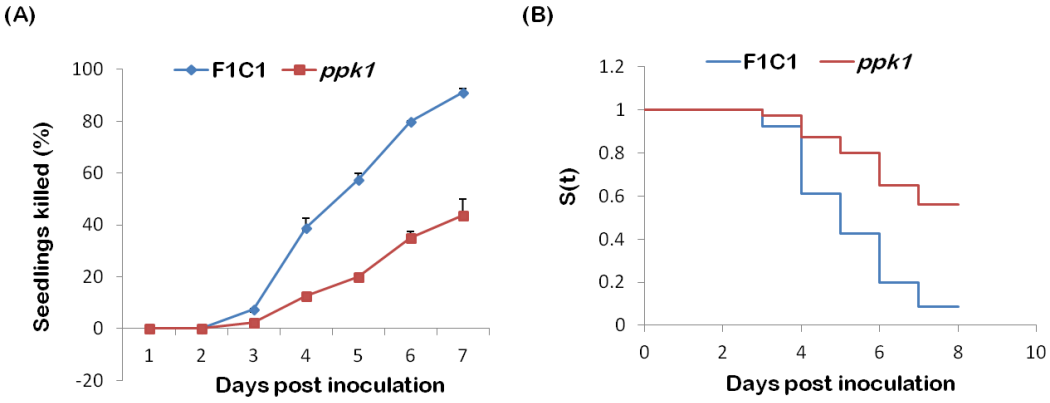


Fig.4.50: Virulence data analysis of *ppk1* insertion mutant by leaf clip inoculation. TRS1019 strain was inoculated in tomato seedlings by leaf dip method. (A) Line graph showing percentage of seedlings killed against days post inoculation (DPI). Each data point is an average of three independent experiments with two replicates. Error bars are depicting standard errors. (B) Kaplan–Meier survival curve depicting survival probability of infected seedlings. The *ppk1* insertion mutant exhibited significant virulence deficiency in compared to the wild type F1C1 ($P < 0.001$; log-rank test).

Further, to attribute the virulence phenotype observed in case of insertion mutant specifically to polyphosphate kinase1 mutant, we recruited deletion mutant of *ppk1* in the virulence study by root inoculation. We found this mutant to be deficient in virulence, suggesting indeed polyphosphate kinase1 is involved in pathogenesis of *R. solanacearum* F1C1 in tomato seedlings. However, number of seedlings killed by this mutant was more compared to insertion mutant; the reason behind is not clear at this time (Fig.4.51).

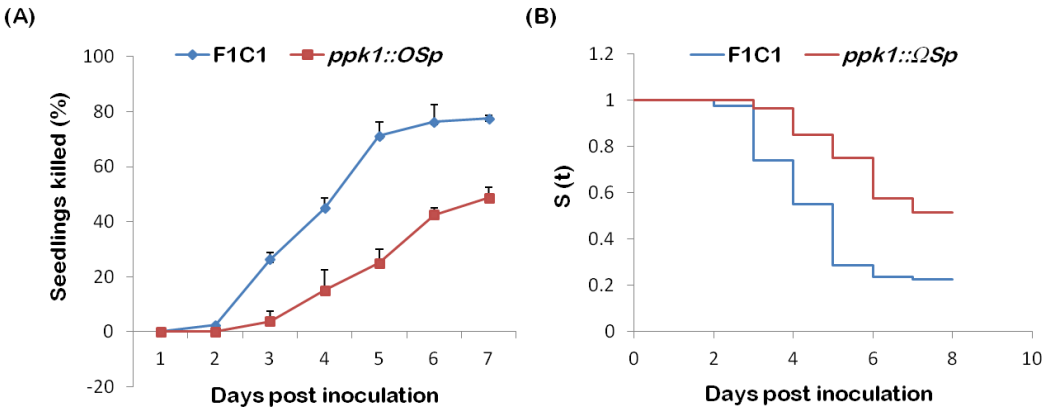


Fig.4.51: Virulence data analysis of *ppk1* deletion mutant by root inoculation. TRS1020 strain was inoculated in tomato seedlings by root dip method. (A) Line graph showing percentage of seedlings killed against DPI. Each data point is an average of three independent experiments. Error bars are depicting standard errors. (B) Kaplan–Meier survival curve depicting survival probability of infected seedlings. The *ppk1* deletion mutant exhibited significant virulence deficiency in compared to the wild type F1C1 ($P < 0.005$; log-rank test).

Inoculating the tomato seedlings with *ppk2* insertion mutant by root dip inoculation, we found a reduced virulence in comparison to wild type F1C1 (Fig.4.52). However, magnitude of virulence deficiency was not as higher as observed in the case of *ppk1* mutants. 88% seedlings were killed in case of F1C1, while it was 76% in case of *ppk2*.

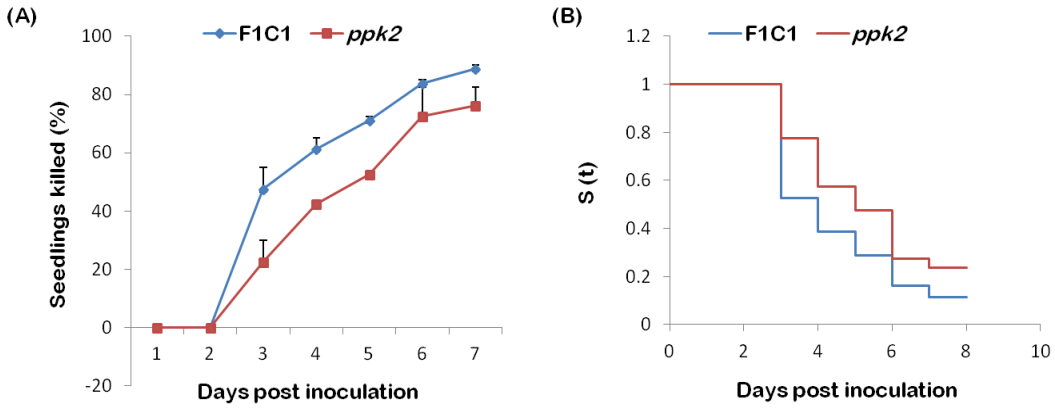


Fig.4.52: Virulence data analysis of *ppk2* mutant by root inoculation. TRS1021 strain was inoculated in tomato seedlings by root dip method. (A) Line graph showing percentage of seedlings killed against DPI. All data point is an average of three independent experiments. Error bars are depicting standard errors. (B) Kaplan–Meier survival curve depicting survival probability of infected seedlings. The *ppk2* mutant exhibited virulence deficiency in compared to the wild type F1C1 ($P < 0.05$; log-rank test).

In the virulence studies of *ppk2* mutant by leaf clip method we could not find significant difference in virulence compared to the wild type. The number of seedlings killed was more compared to root dip inoculation (Fig.4.53).

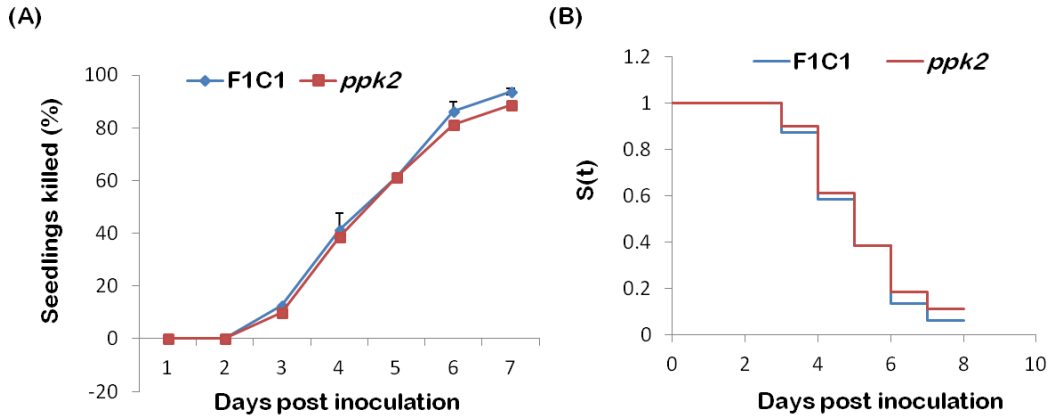


Fig.4.53: Virulence data analysis of *ppk2* mutant by leaf clip inoculation. TRS1021 strain was inoculated in tomato seedlings by leaf clip method. (A) Line graph showing percentage of seedlings killed against days post inoculation (DPI). Each data point is an average of three independent experiments with two replicates. Error bars are depicting standard errors. (B) Kaplan–Meier survival curve depicting survival probability of infected seedlings. The *ppk2* insertion mutant did not exhibit virulence deficiency in compared to the wild type F1C1 ($P > 0.05$; log-rank test).

In a similar way, we studied the virulence phenotype of *ppx* insertion mutant and compared with wild type F1C1. In root inoculation method, in seedlings

inoculated with the mutant, the disease observed was 77% almost like *ppk2*, while in the seedlings inoculated with F1C1; the disease was 88% ($P<0.05$) (Fig.4.54). However, in case of leaf clip inoculation, virulence deficiency was not significant ($P>0.05$), shown in Fig.4.55.

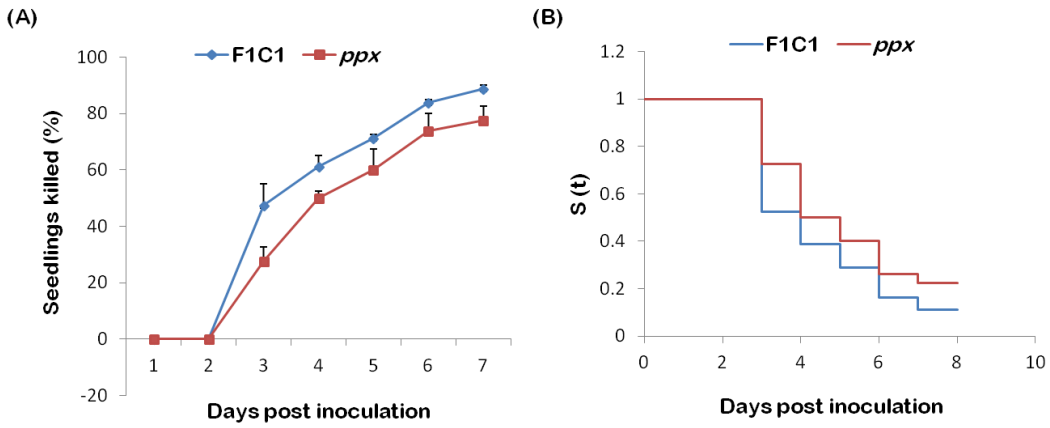


Fig.4.54: Virulence data analysis of *ppx* mutant by root inoculation. TRS1022 strain was inoculated in tomato seedlings by root dip method. (A) Line graph showing percentage of seedlings killed against DPI. Each data point is an average of three independent experiments. Error bars are depicting standard errors. (B) Kaplan–Meier survival curve depicting survival probability of infected seedlings. The *ppx* mutant exhibited virulence deficiency in compared to the wild type F1C1 ($P < 0.05$; log-rank test).

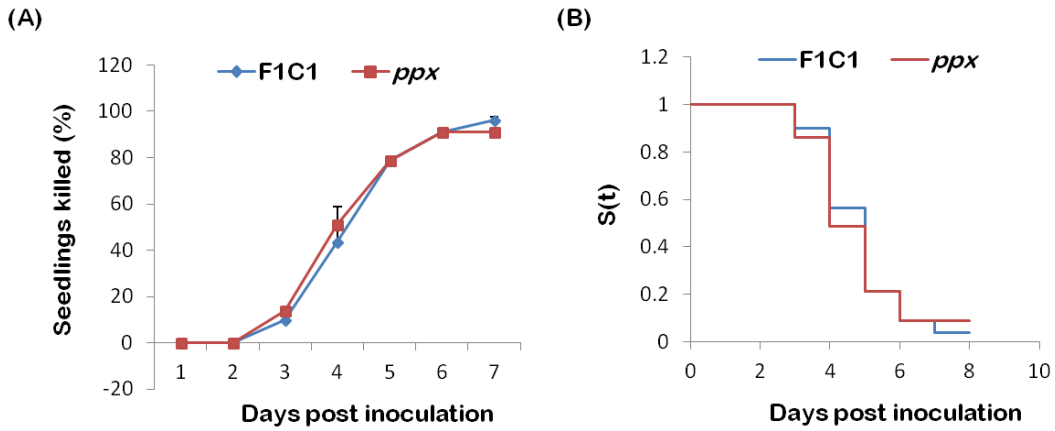


Fig.4.55: Virulence data analysis of *ppx* mutant by leaf clip inoculation. TRS1022 strain was inoculated in tomato seedlings by leaf clip method. (A) Line graph showing percentage of seedlings killed against DPI. Each data point is an average of three independent experiments. Error bars are depicting standard errors. (B) Kaplan–Meier survival curve depicting survival probability of infected seedlings. Virulence deficiency of *ppx* mutant was not significant in compared to the wild type F1C1 ($P >0.05$; log-rank test).

Further, the virulence phenotype of *ppnk* insertion mutant was studied in the same way in tomato seedlings where we found significant virulence deficiency compared to *R. solanacearum* wild type strain, both in leaf clip and root dip inoculation methods. In root inoculation, we observed more than 75% killed seedlings inoculated with wild type, while it was around 45% in the case of *ppnk* mutant, which was statistically significant (P<0.001) (Fig.4.56). Similarly, we found ~ 46-47% of killed seedlings in the case of *ppnk* mutant inoculated by leaf clip whereas it was ~88% with the wild type (Fig.4.57).

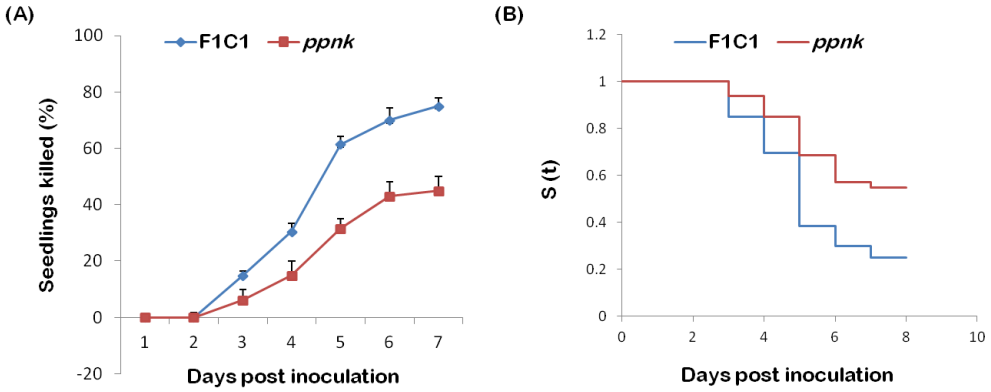


Fig.4.56: Virulence data analysis of *ppnk* mutant by root inoculation. TRS1023 strain was inoculated in tomato seedlings by root dip method. (A) Line graph showing percentage of seedlings killed against DPI. Each data point is an average of three independent experiments. Error bars are depicting standard errors. (B) Kaplan–Meier survival curve depicting survival probability of infected seedlings. The *ppnk* mutant exhibited significant virulence deficiency in compared to the wild type F1C1 (P < 0.001; log-rank test).

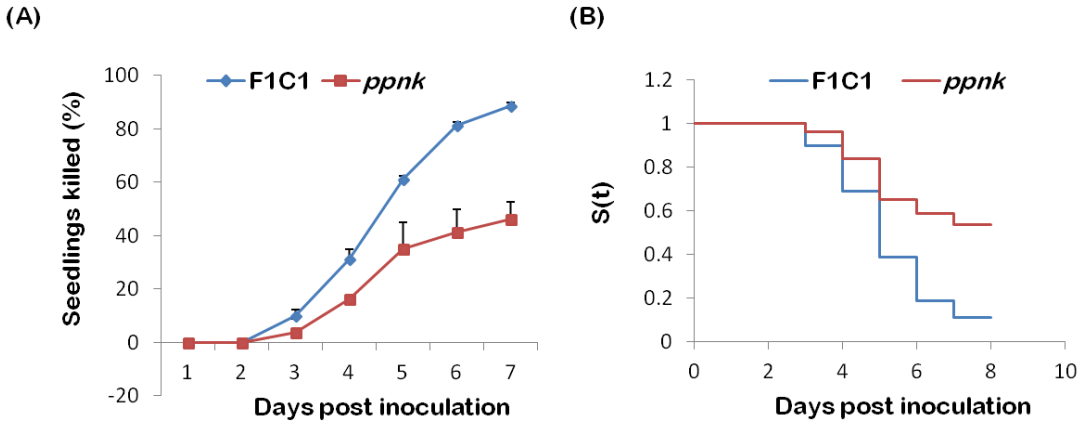


Fig.4.57: Virulence data analysis of *ppnk* mutant by leaf clip inoculation. TRS1023 strain was inoculated in tomato seedlings by leaf clip method. (A) Line graph showing percentage of seedlings killed against DPI. Each data point is an average of three independent experiments. Error bars are depicting standard errors. (B) Kaplan–Meier survival curve

depicting survival probability of infected seedlings. The *ppnk* mutant exhibited significant virulence deficiency in compared to the wild type F1C1 ($P < 0.001$; log-rank test).

Lastly, we were interested to study *ppk1::ppk2* double mutant's disease behavior in tomato seedlings by root inoculation method and compared its phenotype with *ppk1* deletion mutant (the strain in the background of which it was created) and *ppk2* single insertion mutant. An intermediate virulence phenotype was observed in the double mutant which was lower than *ppk2* but higher than *ppk1* deletion mutant (Fig.4.58).

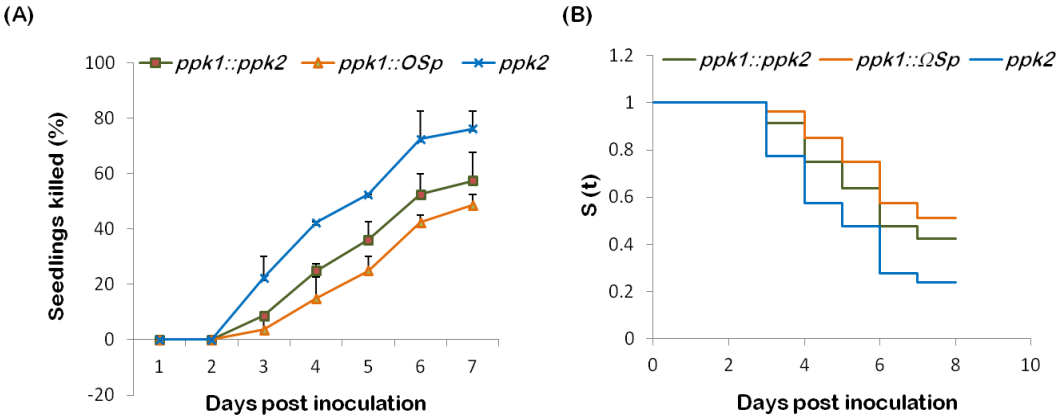


Fig.4.58: Virulence data analysis of *ppk1::ppk2* double mutant by root inoculation. TRS1024 strain was inoculated in tomato seedlings by root dip method. (A) Line graph showing percentage of seedlings killed against DPI. Each data point is an average of three independent experiments. Error bars are depicting standard errors. (B) Kaplan–Meier survival curve depicting survival probability of infected seedlings. The *ppk1::ppk2* double mutant exhibited intermediate virulence phenotype of *ppk1*deletion mutant ($P > 0.05$; log-rank test) and *ppk2* insertion mutants ($P < 0.05$; log-rank test).

4.4.14 PolyP accumulation was detected in *ppx* mutant of F1C1

We were interested to detect PolyP accumulation in wild type F1C1 as well in *ppk1*, *ppk2* and *ppx* mutants and for that we followed a DAPI based approach. DAPI, the widely used fluorescence tag of DNA when binded with PolyP molecules, shift its peak emission wavelength from 415 nm to 550 nm which allows PolyP detection *in vitro* [22]. Using this DAPI based approach; we have done only qualitative detection of PolyP molecules in the studied strains. We could detect presence of PolyP molecules only in *ppx* mutant. We did not observe any emmitance of DAPI in case of wild type or in *ppk1* and *ppk2* mutants (Fig.4.59).

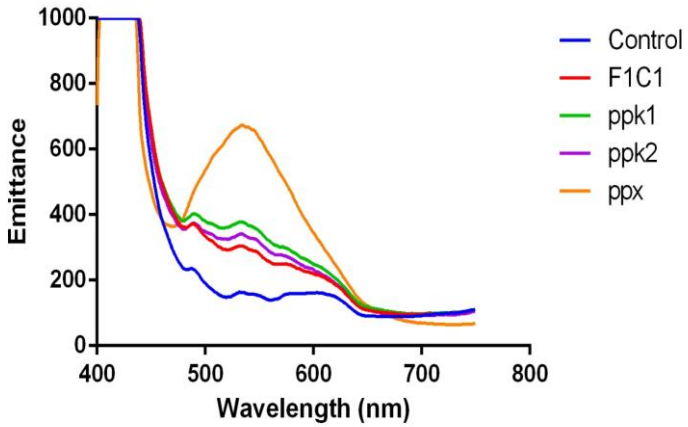


Fig.4.59: Detection of PolyP accumulation in wild type F1C1 and PolyP mutants of F1C1. DAPI fluorescence was scanned in F1C1, *ppk1*, *ppk2* and *ppx* strains after 2 hr of incubation with 2µM DAPI solution. Fluorescence was excited at 415 nm and was scanned against different wavelengths. We observed high DAPI emittance at 550 nm in case of *ppx* mutant unlike others indicate the presence of PolyP molecules.

4.4.15 Complementation study of *ppk1* and *ppnk* mutants

We were interested to do a complementation study in *ppk1* and *ppnk* insertion mutant as these mutants showed significant reduction in swimming motility as well as in virulence. We cloned full length *ppk1* and *ppnk* genes of F1C1 under the control of their own promoter in pNP267 complementation vector and used to transform respective *ppk1* and *ppnk* insertion mutants (as described in section 4.3.11). After the creation of complemented strain of *ppk1* and *ppnk,* we checked their swimming and virulence phenotypes using the same protocol as used for corresponding mutants.

In their swimming motility assay, in case of *ppk1* complemented strain we found that it could restore the motility similar to wild type F1C1(Fig.4.60). However, we could not get back the swimming motility phenotype in case of *ppnk* complemented strain instead showed an intermediate phenotype (Fig.4.61).

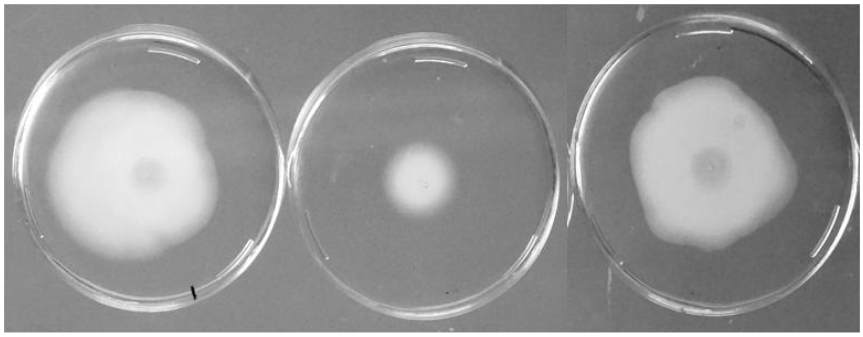


Fig.4.60: Swimming motility assay of *ppk1* complemented strain. Swimming motility was assayed on soft agar plate**s** of (A) wild type F1C1 (B) *ppk1* insertion mutant (C) *ppk1* complemented strain. The *ppk1* mutant showed reduced swimming motility while complemented strain could restore the motility similar to wild type.

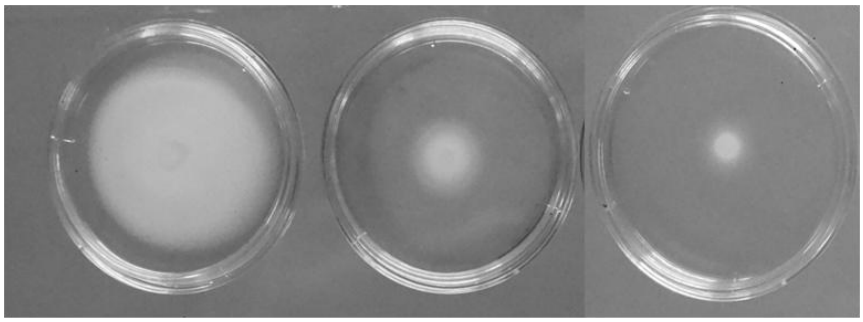


Fig.4.61: Swimming motility assay of *ppnk* complemented strain. Swimming motility was assayed on soft agar plate**s** of (A) wild type F1C1 (B) *ppnk* complemented strain and (C) *ppnk* insertion mutant. The *ppnk* complemented strain could not restore the motility similar to wild type but showed an intermediate motility more than the *ppnk* mutant.

We checked both the *ppk1* and *ppnk* complemented strains for their virulence properties inoculated in tomato seedlings by the root dip inoculation method. The *ppk1*complemented strain showed intermediate virulence of wild type and the mutant (Fig.4.62). However, *ppnk* complemented strain did not restore the virulence deficiency exhibited by the mutant (Fig.4.63).

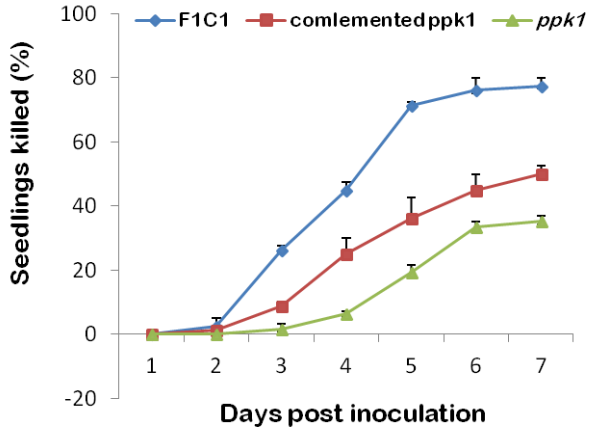


Fig.4.62: Virulence data analysis of *ppk1* complemented strain by root inoculation. TRS1025 strain was inoculated in tomato seedlings by root dip method. (A) Line graph showing percentage of seedlings killed against DPI. The *ppk1* complemented strain exhibited intermediate virulence phenotype of *ppk1* insertion mutant and the wild type. Each data point is an average of three independent experiments. Error bars are depicting standard errors.

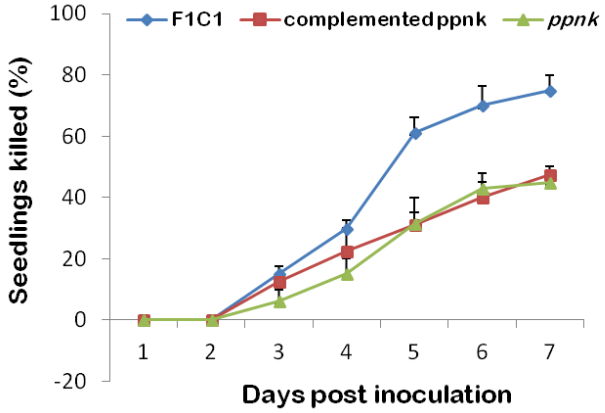


Fig.4.63: Virulence data analysis of *ppnk* complemented strain by root inoculation. TRS1025 strain was inoculated in tomato seedlings by root dip method. (A) Line graph showing percentage of seedlings killed against DPI. The *ppnk* complemented strain exhibited intermediate virulence phenotype of *ppnk* insertion mutant and the wild type. Each data point is an average of three independent experiments. Error bars are depicting standard errors.

4.5 Discussion

In this study, we have characterized the four genes such as *ppk1*, *ppk2*, *ppx* and *ppnk* in *R. solanacearum* by creating insertion mutation. The mutants were found to be proficient for growth, twitching motility, extracellular cellulose activity, eliciting HR in tobacco leaves and stress response to H_2O_2. The mutants exhibited phenotypic changes with regard to virulence, swimming and swarming motility. The

ppk1 and *ppnk* mutants were reduced for virulence inoculated either by the leaf clip or by the root inoculation methods. The *ppk1* mutant was found to be reduced both for the swimming and swarming motility.

Bacterial motility is essential for colonization and the invasion into hosts and is one of the most important virulence functions in pathogenic bacteria [26]. The flagella dependent motility has already been well reported as an important virulence function in phytopathogens such as *Ralstonia solanacearum* [15], *P. syringae* pv. *tabaci* [27], and *Erwinia carotovora* subsp. *carotovora* [28]. Moreover, the involvement of *ppk* in swimming and swarming motility has been reported in several animal pathogenic bacteria such as *H. pylori* [29], *P. aeruginosa* [3] etc. However, dependence of swimming motility on the presence of polyphosphate molecules has not been shown in any phytopathogen. In this study, we found *ppk1*, *ppk2* and *ppnk* mutants to be reduced for swimming motility in comparison to wild type, suggesting the dependence of swimming motility on PolyP molecules in *R. solanacearum* F1C1 strain. We confirmed that the swimming motility deficiency observed was not related with retardation in growth as we had demonstrated that all growth curves were almost similar to the wild type strain. However, swimming motility deficiency was not significant in case of *ppx* mutant. In the swarming motility assay, the *ppk1* mutant was found to be reduced for motility while *ppx* mutant did not show any difference from the wild type. In narrow host range phytopathogen *P. syringae* pv. *tabaci*, the swarming motility of the *ppk* mutant was reduced while swimming motility was unaltered unlike in our case where both swimming and swarming motility was found to be reduced.

Oxidative stress tolerance and survival in pathogens like *Erwinia chrysanthemi* and *E. carotovora* subsp. *carotovora* are important virulence factors [30,31] and in *P. syringae* pv. *Tabaci 6605;* PolyP has been shown to play crucial role in this regard [32]. However, in case of *R. solanacearum* we did not find significant role of PolyP in oxidative stress tolerance induced by hydrogen peroxide in nutrient rich culture plate. In another preliminary study, we observed sensitivity of polyphosphate mutants to same oxidant grown in minimal media condition which needs further detail investigation.

Several studies have showed the involvement of PolyP in virulence properties of *Salmonella, Shigella, H. pylori, C. jejuni, P. aeruginosa,,* including the phytopathogen *P. syringae* pv. *Tabaci 6605* [4,29,32,33,34,35]. Our virulence studies of PolyP metabolism mutants in tomato seedlings both by root inoculation and leaf clip inoculation methods revealed that PolyP molecules play an important role in pathogenesis of *R. solanacearum* F1C1 inside the host.

We did not observe a consistent virulence phenotype of *ppnk* with regard to the complemented strain. One of the explanations might be the phenotype is resulted due to some mutation in other locus that is responsible for the virulence. In *R. solanacearum* genome the *ppnk* gene is in operon with *recN* gene hence chance of polar mutation effect cannot be eliminated. In future we will be interested to create multiple mutants of *ppnk* and study their phenotypes. This will be followed by complementation study with the developed plasmid. It is also pertinent to note that swimming motility is not a consistent phenotype to study in *R. solanacearum*. Therefore, complementation study in virulence phenotype will be more applicable for understanding the role of *ppnk* in *R. solanacearum* F1C1.

Considering the phenotypes of the *ppk1* mutant, PPK1 seems to be an important enzyme for the PolyP metabolism in this bacterium. Quantification of PolyP in different mutants will be an important future study to get a detailed insight into PolyP role in different functions of this bacterium.

Leiberman (1888) found out this multifunctional PolyP and since then it has been extensively studied [36]. Various functions of polyphosphate kinases; synthesizing enzyme of this molecule have been already reported in the literature [3,34,35,37,38] however we believe present study is the first demonstration of role of polyphosphate kinase function in broad host range phytopathogenic bacteria, *R. solanacearum*.

References:

[1] Kornberg, A. Inorganic Polyphosphate: Toward making a forgotten polymer unforgettable, *Journal of bacteriology*, 177:491–496, 1995.

[2] Ogawa, N., Tzeng, C. M., Fraley, C. D., and Kornberg, A. Inorganic polyphosphate in Vibrio cholerae: genetic, biochemical, and physiologic features. *Journal of bacteriology*, 182(23):6687–6693, 2000.

[3] Rashid, M. H., and Kornberg, A. Inorganic Polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeroginosa*. *PNAS*, 97:4885–4890, 2000.

[4] Kim, K. S., Rao, N. N., Fraley, C. D., and Kornberg, A. Inorganic polyphosphate is essential for long-term survival and virulence factors in *Shigella* and *Salmonella* spp. *Proceedings of the National Academy of Sciences*, 99(11):7675–7680, 2002.

[5] Tan, S., Fraley, C. D., Zhang, M., Dailidiene, D., Kornberg, A., and Berg, D. E. Diverse phenotypes resulting from polyphosphate kinase gene (ppk) inactivation in different strains of Helicobacter pylori. *Journal of Bacteriology*, 187:7687–95, 2005.

[6] Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P., Dow, M. A. X., Verdier, V., Beer, S.V., Machado, M.A., Toth, I. A. N., Salmond, G., and Foster, G. D. Top 10 plant pathogenic bacteria in molecular plant pathology. *Molecular Plant Pathology,* 13: 614–629, 2012.

[7] Kumar, R., Barman, A., Jha, G., and Ray, S. K. Identification and establishment of genomic identity of *Ralstonia solanacearum* isolated from a wilted chilli plant at Tezpur, North East India. *Current Science,* 105:1571–1578, 2013.

[8] Monteiro, F., Solé, M., van Dijk, I., and Valls, M. A chromosomal insertion toolbox for promoter probing, mutant complementation, and pathogenicity studies in Ralstonia solanacearum. *Molecular plant-microbe interactions*, 25(4):557–568, 2012.

[9] Inoue, H., Nojima, H., and Okayama, H. High efficiency transformation of *Escherichia coli* with plasmids, *Gene*, 96:23–28, 1990.

[10] Cunnac, S., Occhialini, A., Barberis, P., Boucher, C., and Genin, S. Inventory and functional analysis of the large Hrp regulon in *Ralstonia solanacearum*: identification of novel effector proteins translocated to plant host cellsthrough the type III secretion system, *Molecular Microbiology*, 53:115–128, 2004.

[11] Gonzalez, A., Plener, L., Restrepo, S., Boucher, C., and Genin, S. Detection and functional characterization of a large genomic deletion resulting in decreased pathogenicity in *Ralstonia solanacearum* race 3 biovar 2 strains. *Environmental microbiology*, 13(12):3172–3185, 2011.

[12] Miller, J.H. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pages 352–355, 1972.

[13] Tanaka Y., Takahashi, H., Simidu, U., and Kimura, B. Design of a new universal realtime PCR system targeting the *tuf* gene for the enumeration of bacterial counts in food, *Journal of Food Protection*, 73:670–679, 2010.

[14] Prentki, P. and Krisch, H. M. In vitro insertional mutagenesis with a selectable DNA fragment, *Gene*, 29:303–313, 1984.

[15] Tans-Kersten, J., Brown, D., and Allen, C. Swimming Motility, a Virulence Trait of *Ralstonia solanacearum*, Is Regulated by FlhDC and the Plant Host Environment. *Molecular Plant-Microbe Interactions*, 17(6):686–695, 2004.

[16] Kai Wu et al. Competitive use of root exudates by Bacillus amyloliquefaciens with *Ralstonia solanacearum* decreases the pathogenic population density and effectively controls tomato bacterial wilt. *Scientia Horticulturae*, 218:132–138, 2017.

[17] Ray, S. K., Kumar, R., Peeters, N., Boucher, C., and Genin, S. rpoN1, but not rpoN2, is required for twitching motility, natural competence, growth on nitrate, and virulence of *Ralstonia solanacearum*. *Frontiers in Microbiology*, 6:229, 2005.

[18] Teather, R. M. and Wood, P. J. Use of congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen, *Applied. Environmental Microbiology*, 43:777–780, 1982.

[19] Singh, N., Phukan, T., Sharma, P. L., Kabyashree, K., Barman, A., Kumar, R., Sonti, R. V., Genin, S., and Ray, S. K. An innovative root inoculation method to study *Ralstonia solanacearum* pathogenicity in tomato seedlings. *Phytopathology*, 108:436–442, 2018, DOI:10.1094/PHYTO-08-17-0291-R.

[20] Kumar, R., Barman, A., Phukan, T., Kabyashree, K., Singh, N., Jha, G., Sonti, R. V., Genin, S., and Ray, S. K. *Ralstonia solanacearum* virulence in tomato seedlings inoculated by leaf clipping. *Plant Pathology*, 66:835–841, 2017.

[21] Kaplan, E. L. and Meier, P. Nonparametric estimation from incomplete observations. *Journal of the American Statistical Association*, 53:457–81, 1958.

[22] Aschar-Sobbi, R., Abramov, A. Y., Diao, C., Kargacin, M. E., Kargacin, G. J., French, R. J., and Pavlov, E. High sensitivity, quantitative measurements of polyphosphate using a new DAPI-based approach. *Journal of fluorescence*, 18(5):859–866, 2008.

[23] Liu, H., Kang, Y., Genin, S., Schell, M.A. and Denny, T.P. Twitching motility of *Ralstonia solanacearum* requires a type IV pilus system. *Microbiology,* 147:3215–3229, 2001.

[24] Kang, Y., Liu, H., Genin, S., Schell, M.A., and Denny, T.P. *Ralstonia solanacearum* requires type 4 pili to adhere to multiple surfaces and for natural transformation and virulence. *Molecular Microbiology*, 46(2):427–437, 2002.

[25] Liu, H., Zhang, S., Schell, M. A., and Denny, T. P. Pyramiding unmarked deletions in *Ralstonia solanacearum* shows that secreted proteins in addition to plant cell-wall-degrading enzymes contribute to virulence, *Molecular Plant-Microbe Interactions,* 18:1296–1305, 2005.

[26] Chua, K. L. Flagella are virulence determinants of *Burkholderia pseudomallei*. *Infection and Immunity*, 71:1622–1629, 2003.

[27] Ichinose ,Y., Shimizu, R., Ikeda, Y., Taguchi, F., Marutani, M., Mukaihara, T., Yoshishige, I., Kazuhiro T., and Tomonori S. Need for flagella for complete virulence of Pseudomonas syringae pv. tabaci: genetic analysis with flagella-defective mutants 6fliC and 6fliD in host tobacco plants. *Journal of General Plant Pathology,* 69:244–9, 2003.

[28] Hossain, M. M., Shibata, S., Aizawa, S., Tsuyumu, S. Motility is an important determinant for pathogenesis of *Erwinia carotovora subsp. carotovora. Physiological and Molecular Plant Pathology*, 66:134–43, 2005.

[29] Ayraud, S., Janvier, B., Labigne, A., Ecobichon, C., Burucoa, C., and Fauchere, J. L. Polyphosphate kinase: a new colonization factor of Helicobacter pylori. *FEMS microbiology letters*, 243(1):45–50, 2005.

[30] Hassouni, M.E., Chambost, J.P., Expert, D., Gijsegem, F.V., Barras, F. The minimal gene set member msrA, encoding peptide methionine sulfoxide reductase, is a virulent determinant of the plant pathogen Erwinia chrysanthemi. *Proceedings of the National Academy of Sciences*, 96:887–92, 1999.

[31] Andersson, R.A., Koiv, V., Norman-Setterblad, C., Pirhonen, M. Role of RpoS in virulence and stress tolerance of the plant pathogen Erwinia carotovora subsp. carotovora. *Microbiology*, 145:3547–56, 1999.

[32] Hossain, M. M., Tani, C., Suzuki, T., Taguchi, F., Ezawa, T., and Ichinose, Y. Polyphosphate kinase is essential for swarming motility, tolerance to environmental

stresses, and virulence in Pseudomonas syringae pv. tabaci 6605. *Physiological and Molecular Plant Pathology*, 72(4-6):122–127, 2008.

[33] McMeechan, A., Lovell, M. A., Cogan, T. A., Marston, K. L., Humphrey, T. J., and Barrow, P. A. Inactivation of ppk differentially affects virulence and disrupts ATP homeostasis in Salmonella enterica serovars Typhimurium and Gallinarum. *Research in microbiology*, 158(1):79–85, 2007.

[34] Candon, H. L., Allan, B. J., Fraley, C. D., and Gaynor, E. C. Polyphosphate kinase 1 is a pathogenesis determinant in Campylobacter jejuni. *Journal of bacteriology*, 189(22):8099–8108, 2007.

[35] Rashid, M. H., Rumbaugh, K., Passador, L., Davies, D. G., Hamood, A. N., Iglewski, B. H., and Kornberg, A. Polyphosphate kinase is essential for biofilm development, quorum sensing, and virulence of *Pseudomonas aeruginosa. PNAS*, 97 (17):9636–9641, 2000.

[36] Lieberman, L. Uber das Nuclein der Hefe und Kunstliche Dar-stellung eines Nucleus Eiweiss und Metaphosphatsaure. *Ber. Chem-Ges.*, 21:598–607, 1888.

[37] Kornberg, A., Rao, N.N., and Aulit-Riche, D. Inorganic polyphosphate: a molecule of many functions. *Annual Review of Biochemistry*, 68:89–125, 1999.

[38] Fraley, C.D., Rashid, M.H., Lee, S.S., Gottschalk, R., Harrison, J., Wood, P.J., Brown, M.R. and Kornberg, A. A polyphosphate kinase 1 (ppk1) mutant of *Pseudomonas aeruginosa* exhibits multiple ultrastructural and functional defects. *Proceedings of the National Academy of Sciences*, 104(9):3526–3531, 2007.