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Isolation, cloning and characterization of *phlA* gene from an indigenous *Pseudomonas* strain from Indian soil

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ABSTRACT: *Pholoroglucinol is a phenolic polyketide compound produced by gram negative bacteria Pseudomonas flourescens. Phloroglucinol is known to inhibit many soil fungi and bacteria. This property is accountable for role of fluorescent Peusdomonads as a potent biological control agent. The genes involved in the biosynthesis of 2, 4-DAPG are organised into an operon of six genes viz; phlA, phlB, phlC, phlD, phlE and phlF. Activity of phlA, phlB, phlC and phlD is necessary for the synthesis of MAPG and its conversion to 2, 4-DAPG. Thesegenes are highly conserved within the genus Pseudomonas and are reported to belong to a novel type of PKS. phlA gene sequence shows deviations from normal polyketide biosynthetic genes and current accumulating data regarding phlA gene is insignificant owing to its function. The present study focuses on cloning of phlA gene from an Indian strain of Pseudomonas flourescens and its sequence analysis for deep insight into its function.*

Keywords: *Phloroglucinol, polyketide, biosynthesis, biological control, operon.*

INTRODUCTION

Phloroglucinols are phenolic plant metabolites with broad-spectrum antiviral, antibacterial, antifungal, antihelminthic, and phytotoxic properties. 2, 4-Diacetylphloroglucinol (2, 4-DAPG), produced by certain plant-associated fluorescent *Pseudomonads* of worldwide origin (Dowling *et al*. 1994, Keel *et al.* 1996), is known to have antagonistic activity against a variety of root and seedling pathogens of plants (Thomashow and Weller 1994). It has gained attention for its role as a biological control agent in modern agricultural practices against root diseases (Cook *et al*. 1995, Sherrif *et al.* 1998). DAPG negative mutants of *Pseudomonas fluorescens* and non-producing strains are reported to show biocontrol activity upon transfer of DAPG biosynthetic plasmids, thus supporting the role of 2, 4-DAPG in plant protection (Bonsall *et al*. 1997; Raaijmakers *et al*. 1998).

2, 4-DAPG inhibits a wide range of fungi and bacteria and show strong biocontrol activity against damping-off, root rot, and wilt diseases caused by soil-borne fungal pathogens, and play a key role in the natural suppression of *Gaeumannomyces graminis* var. *tritici*, known as take-all decline, the fungal pathogen that causes take-all disease of wheat. The biosynthetic gene cluster for 2, 4 DAPG production was found to be conserved among different species of DAPG producing *Pseudomonad*'s when analysed in samples from the soils that are naturally suppressive to take-all of wheat, black root rot of tobacco, and tomato wilt caused by the fungal pathogens *Gaeumannomyces graminis*, *Thielaviopsis basicola*, and *Fusarium oxysporum*, respectively (Raaijmakers *et al*, 1997; Weller *et al*, 1998).

Genes essential for biosynthesis of 2, 4-DAPG are organized on a 6.5 kb DNA fragment in *P. fluorescens* Q2-87. The biosynthetic operon includes *phlA*, *phlB*, *phlC*, and *phlD* transcribed from a promoter upstream of *phlA* (Thomashow 1996). *PhlD* is responsible for the production of monoacetylphloroglucinol (MAPG), and *PhlA, PhlB* and *PhlC,* are necessary to convert MAPG to 2, 4-DAPG. The biosynthetic operon is flanked on either side by *phlE* and *phlF*, which code respectively for putative efflux (transport) and repressor proteins. *PhlD* is responsible for the production of monoacetylphloroglucinol (MAPG), and *PhlA, PhlB* and *PhlC* are necessary to convert MAPG to 2, 4-DAPG (Bangera *et al* 1996; Shanahan *et al* 1993) suggests the function of these proteins as a complex to facilitate substrate transfer reactions. *phlA*, *phlC*, and *phlB* are found in archaebacterial genomes but not in eubacteria, whereas *phlD* homologous are

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present in the eubacterial genomes of *Mycobacterium* and as well as in plants. Sensor kinase GacS (formerly designated LemA) and the cognate response regulator *GacA* together form a 2 component system and are essential for the synthesis of 2, 4-DAPG (Blumer *et al* 1999; Corbel *et al* 1995; Laville *et a,* 1998; Whisteler *et al* 1998).

Expression studies have revealed that the product of all the four genes *phlA*, *phlB*, *phlC* and phlD are necessary for the production of MAPG and 2, 4 DAPG. Mutation in a single gene will lead to faulty product. Products of these genes resemble neither type I nor type II PKS enzyme systems. Rather, *PhlD* shows similarity to plant chalcone synthases, indicating that phloroglucinol synthesis is mediated by a novel kind of PKS (Voisard *et al* 1994; Whisteler *et al* 1998; Yuan *et al* 1998).

The production of 2, 4-DAPG is stimulated by glucose in many strains (Duffy and Deffago 1997) or by sucrose or ethanol in a few strains (Shanahan *et al* 1993; Yuan *et al* 1998). Among the products of all four genes viz; *phlA*, *phlB*, *phlC* and phlD; only the expression of *phlA* is autoinduced by 2, 4-DAPG and strongly repressed by the bacterial extracellular metabolites salicylate and pyoluteorin as well as by the fungal metabolite fusaric acid. But its exact individual role is yet to be resolved. Our work focuses on the cloning of *phlA* gene from an Indian strain of *Pseudomonas fluorescens* to get a deep insight of its function. This will help modulate (up-regulate/down-regulate) the synthesis of 2, 4-DAPG in response to potent fungal and bacterial pathogens.

MATERIAL AND METHODS

Genomic DNA isolation

Two ml culture of *Pseudomonas fluorescens* was pelleted out (centrifuged at 10,000 rpm for 5 minutes) twice. The pellet was resuspended in 500µl of TE buffer (pH 8.0) + glucose (50mM), 50µl of lysozyme (10mg/ml) and 5µl RNAse (100mg/ml) and was incubated at 37°C for 30 minutes. Next, 60µl of 10% SDS and 50µl of proteinase k (10mg/ml) was added, mixed and incubated at 55°C for two hours. After incubation, equal volume of Tris-saturated phenol (pH 8.0) was added and solution was vortexed for 20-30 seconds. The mixture was then centrifuged at 10,000 rpm for 10 minutes. To the supernatant equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added. The extraction was repeated until no protein precipitate was obtained at interface. To the aqueous phase 0.1 volume of 3M sodium acetate (pH 5.5) was added. It was mixed gently and 2.5 volume of the ice cold ethanol was added and mixed by vortexing for a few seconds. The mixture was incubated at -80°C for one hour. The tubes were centrifuged at 10000 rpm for 5 minutes at 4°C. Supernatant was discarded. To pelleted DNA, equal volume (aqueous) of 70% ethanol added and centrifuge at 14,000 rpm for 5 minutes. Supernatant was discarded; the pellet was dried and dissolved in 100µl of 0.1X TE buffer.

PCR and Cloning

Cloning of the gene encoding *phlA* was achieved by polymerase chain reaction (PCR). *phlA* gene was amplified from the genomic DNA of *Pseudomonas fluorescens*. For amplification of *phlA* gene, forward and reverse primers were generated using PRIMER 3 tool. Primer {(*phlA*F: TGCCGGGTTTGGAAGTATAG and *phlA*R: TGGCAGGTGAAGAAAGGAAT)} was designed using the nucleotide sequence of *Pseudomonas fluorescens* (U41818) available at EMBL database. The reaction mixture consisted of 10 pmol of each primer, 50 ng of template DNA, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1.25 unit of *Taq* DNA polymerase in a 100-ìl volume. The thermal cycling was performed after an initial denaturing cycle of 5 min at 95°C. Then 30 cycles were completed using the following temperature profiles: denaturation at 95°C for 1 min, annealing for 30 s at 47.1°C, and extension for 1 min at 72°C. PCR products were cloned into the pCR2.1/ TOPO vector (Invitrogen) according to the manufacturer's instructions. The purified PCR product was ligated in pCR2.1 cloning vector (available in the linearised form). This ligated mix was kept at 4°C overnight and used for transformation into *E.coli.* The transformed colonies (white in colour) obtained after overnight incubation at 37°C were picked and streaked onto fresh LA-carbenicillin plates. Positive colonies were confirmed by colony PCR with gene specific primers and restriction with Eco*RI* enzyme. The amplified and restricted DNA sample was analyzed on 1% agarose gel.

The complete DNA sequence was determined by Sanger sequencing. Vector pCR2.1-based primers, universal forward and reverse, M13F and M13R primers were used in sequencing reactions. *phlA* based primers were also used for clarifying ambiguities. The final sequence was determined from both strands and comparison of *phlA* nucleic acid and amino acid sequences were performed.

RESULT AND DISCUSSION

Among varied bacterial kingdom, *Pseudomonad*s are the best and only natural sources of phloroglucinol. Fluorescent *Pseudomonas* spp. play an important role in the biological control of many plant pathogens such as damping off of sugar beet, black root rot of tobacco etc. The antibiotic properties of DAPG against soil-borne fungal pathogens have been known for more than 100 years, but research advances in the last two decades provide significant insights into the mechanism of their biosynthesis and antibiotic actions.

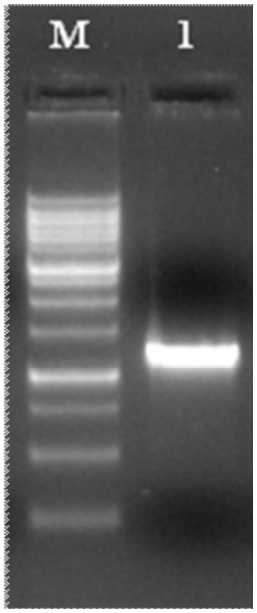

Figure 1: PCR amplification of putative *phlA* gene from genomic DNA of *Pseudomonas fluorescens*. Lane M: 1 kb DNA ladder (Fermentas); Lane 1: ~1.2 kb amplicon of *phlA* gene

To date, the complete genomic sequences of several *P. fluorescens* strains are available. The genomic information provided new opportunity to advance knowledge of DAPG biosynthesis in our isolated *Pseudomonas* strain. Production of DAPG is regulated by complex genetic mechanisms as well as being modulated by multiple biotic and abiotic factors. The *phl* locus, which encodes the biosynthetic and regulatory genes for DAPG was discerned in this study by cloning the *phlA* gene.

PCR with gene specific primer using genomic DNA of *Pseudomonas* resulted in an expected amplicon of size ~1.2 kb (Figure 1). The amplified PCR product was cloned in pCR2.1/TOPO vector and randomly 14 colonies were used for colony PCR screening for the presence of the *phl*A gene (Figure 2, Lane 6). Presence of *phl*A gene was further confirmed by restriction digestion with *Eco*RI enzyme that released the expected fragment of ~1.2 kb (Figure 3).

The newly cloned sequence of *phlA* consisted of 1201 nucleotides with an open reading frame from 36 to 1,118. The longest ORF of the *phlA* gene was found to 1080bp. Based on the blast results the *phlA* gene was found to be full length coding 360 amino acids. The translated nucleotide sequence also depicts the start codon. This is consistent with the other reports (Bangera and Thomashow 1999). A putative ATG start codon for *phlA* is not preceded by a consensus ribosome binding site. The sequence identity of this new cloned sequence is in contrast to previously cloned *phlA* genes from different *Pseudomonas* species like *Pseudomonas* sp. CM1'A2, *Pseudomonas* sp. K94.37, *Pseudomonas* sp. PILH1, *Pseudomonas* sp. P12, *Pseudomonas* sp. K93.2 and more, which share about 90-95% amino acid sequence identity (Figure 4).

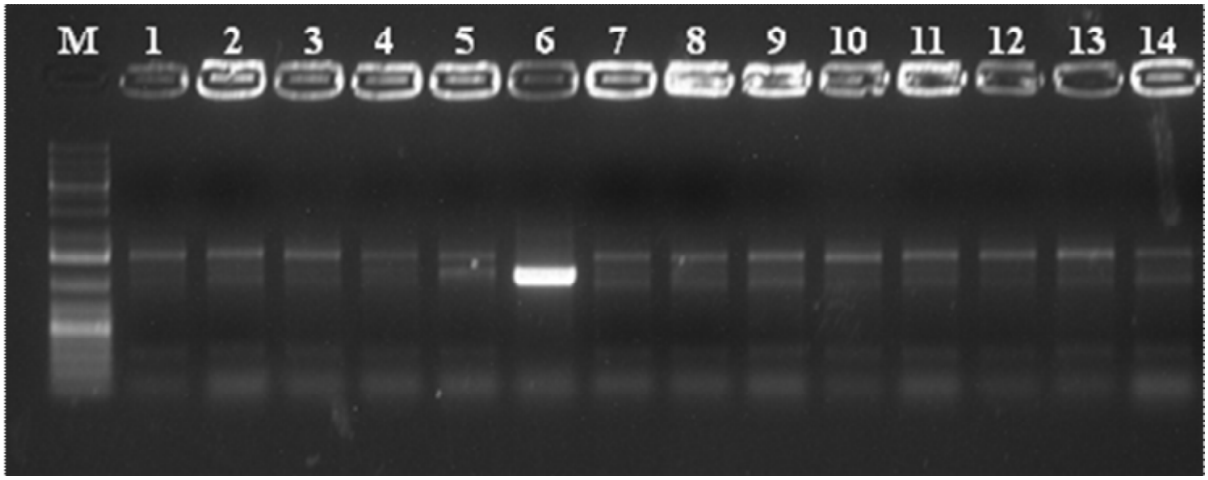

Figure 2: Colony PCR of positive colonies with gene specific primers. Lane M: 1 Kb plus DNA ladder (Fermentas); Lane 1-14: PCR amplified product. Only colony 6 yielded an amplicon of ~1.2 kb

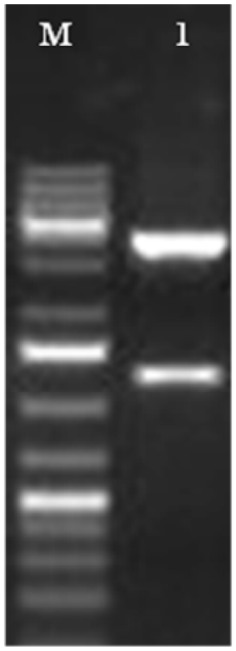

Figure 3: Restriction digestion of putative *phlA* clone plasmid DNA confirmed by colony PCR. Lane M: 1 kb Plus DNA ladder (Fermentas); Lane 1: Restriction digestion with Eco*RI* released a fragment of ~1.2 kb of *phlA* gene and ~4 kb fragment of pCR2.1 vector backbone

The putative product of *phlA* shows 26% identity and 48% similarity to the 33-kDa *fabH* gene product â-ketoacyl-acyl carrier protein synthase III (KAS III) from *E. coli* (Tsay et al 1992). An imperfect phosphopantetheine-binding motif (IGADTINRNTAPGDL) was identified at residue 143 in which a threonine (underlined) replaces the conserved pantotheine-binding serine residue. It also lacks the active site cysteine residue.

Cloned *phlA* gene shows significant homology with other known genes, indicating common evolutionary origins and suggesting role in mechanisms for the gene regulation, synthesis, and export of DAPG. The deduced product (360 amino acids, 37.9 kDa) of the *phlA* gene showed 93% identity with its homolog *phlA*, the first gene product of the 2, 4-DAPG biosynthetic operon in strain Q2-87 (Bangera and Thomashow 1999) (Figure 5).

The *phlA* gene along with *phlB* and *phlC*, is vital for the production of 2,4 DAPG from MAPG (Shanahan et al 1993). It has been reported that mutation in any one genes give rise to a common phenotype (Kletzin and Adams 1996). Although its individual role as chain length factor, perhaps through interactions with *phlD* still needs to be

YP_004353605.1	2,4-diacetylphloroglucinol biosynthesis protein [Pseudomonas bras	630	630	99%	0.0	97%
AAY86548.1	PhlA [Pseudomonas fluorescens]	627	627	99%	0.0	97%
AAM27405.1	PhlA [Pseudomonas fluorescens]	624	624	99%	0.0	96%
AAB48109.1	PhlA [Pseudomonas fluorescens Q2-87] >gb|ADG03653.1| PhlA [Ps	601	601	99%	0.0	93%
BAD00178.1	PhlA [Pseudomonas fluorescens]	582	582	99%	0.0	90%
ABO30419.1	PhlA [Pseudomonas sp. CM1'A2] >gb|ABO30422.1| PhlA [Pseudom	565	565	91%	0.0	97%
ABO30426.1	PhlA [Pseudomonas sp. K94.37] >gb|ABO30429.1| PhlA [Pseudomo	565	565	91%	0.0	97%
ABO30420.1	PhlA [Pseudomonas sp. PILH1] >gb|ABO30421.1| PhlA [Pseudomor	565	565	91%	0.0	97%
ABO30427.1	PhlA [Pseudomonas sp. P12]	563	563	91%	0.0	96%
ABO30424.1	PhlA [Pseudomonas sp. K93.2]	563	563	91%	0.0	96%
AEB78028.1	PhlA [Pseudomonas sp. K93.52]	563	563	91%	0.0	97%
AED70037.1	PhlA [Pseudomonas sp. S8-151]	562	562	91%	0.0	96%

Figure 4: Blast result of novel PHLA protein showing similarity with other species of *Pseudomonas*.

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           1       10        20        30        40        50        60        70        80        90       100       110       120       130
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
DAPG       MNKVGIVSYGAGIPVCRLKVDDVIQVWKNTDLSLVKGQLGVIERAVLQPDEDVITLGVLAAQRALDEAPPCSLEALYLGTCTNPYDSRASAAIILEMLGCGYDAFCADVQFAGKSGTSALQIAYALVASG
phlA       MNKVGIVSYGSGIPVCRPKVDDVIQVWKNTDLSLVKDQLGVIERAVLQPDEDVITLGVLAAQRALDKAPPCSLEALYLGTCTNPYDSRASAAIILEMLGCGYDAFCADVQFAGKSGTSALQIAYALVASG
Consensus  MNKVGIVSYGaGIPVCRlKVDDVIQVWKNTDLSLVKdQLGVIERAVLQPDEDVITLGVLAAQRALDeAPPCSLEALYLGTCTNPYDSRASAAIILEMLGCGYDAFCADVQFAGKSGTSALQIAYALVASG

           131     140       150       160       170       180       190       200       210       220       230       240       250       260
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
DAPG       MVGNALAYGADTINRNTAPGDLTESYAGAGAAALLLGTENVIAHFDASFSCAADVADNIRPQGDRYIRSGHGLGPDKNSIGLEDQTRRAASGLMAKIHAQADDFDYVVFQQNLVSTPYSLGKHLGFTKAQ
phlA       MVGNALAYGADTINRNTAPGDLTESYAGAGAAALLLGTENVIAHFDASFSCAADVAGNIRPQGDRYIRSGHGLGSDKNSIGLEDQTRRAASGLMAKIHAQADDFDYVVFRQNLVSTPYSLGKHLGFTKAQ
Consensus  MVGNALAYGADTINRNTAPGDLTESYAGAGAAALLLGTENVIAHFDASFSCAADVAdNIRPQGDRYIRSGHGLGpDKNSIGLEDQTRRAASGLMAKIHAQADDFDYVVFrQNLVSTPYSLGKHLGFTKAQ

           261     270       280       290       300       310       320       330       340       350       360
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
DAPG       IEPGIYAQSVGDAGAASPLLGLVNVLDQARPGERILVVSYGFGAGSDAIALTVTDAIEAYQKTNVPLRTQLEDKYYVDYRTSIKYEFKYLRPDYALTAYL
phlA       IEPGIYAQSVGDAGAAGPLLGLVNVLDQARPGERILLVSYGFGAGSDAIALTVTDAIEAYQKTNVPLRTLLEDKYYVDYGTSIKYEFKYLRPDYALTAYL
Consensus  IEPGIYAQSVGDAGAAgPLLGLVNVLDQARPGERILlVSYGFGAGSDAIALTVTDAIEAYQKTNVPLRTqLEDKYYVDYrTSIKYEFKYLRPDYALTAYL
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Figure 5: Pairwise sequence alignment of isolated PHLA protein with reported DAPG producing PHLA protein from *Pseudomonas* data base.

enlightened. Sequence analysis gives partial information about *phlA*. Substitution of serine with threonine results in imperfect phosphopantetheine-binding motif which leads to loss of function (Jaworski *et al.* 1989). Absence of essential cystein present in the active site of condensing enzymes from *phlA* makes it integral to the basic architecture of bacterial type II PKSs, where they help to determine the chain length of the poly-â-ketone intermediate prior to cyclization (Hutchinson and Fujii 1995). This reflects the significance of *phlA* gene in the DAPG biosynthetic operon.

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