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Cloning and characterization of phloroglucinol biosynthetic gene *phl*C from an Indian strain of *Pseudomonas fluorescens*

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Phloroglucinol or 2,4-diacetyl phloroglucinol (DAPG) is a polyketide compound produced by gram negative soil bacteria *Pseudomonas*. It shows broad spectrum antibacterial and antifungal properties against soil-borne plant pathogens. In *Pseudomonas* spp., genes for biosynthesis of 2,4-DAPG are localized in *phlABCD* operon. All the four genes in *phlABCD* operon are indispensable and DAPG synthesis is attenuated even in the absence of one of the genes. In the present study, we identified and cloned *phlC* gene from an Indian strain of *Pseudomonas* and analyzed its sequence. The structural details of the PHLC protein was generated by three-dimensional homology modelling. Additionally, stereo-chemical properties of PHLC were analyzed by Ramachandran plot analysis and the generated model was validated by PDBsum. Our results demonstrate that the cloned PHLC protein contains structural features typical of a condensing enzyme involved in polyketide synthesis.

Keyword: PGPR, *phl*ABCD, Phloroglucinol, Polyketide, Protein model database (PMDB), Ramchandran plot

Phloroglucinol is known to inhibit the growth of a wide range of fungi and bacteria with strong biocontrol activity against damping-off, root-rot, and wilt diseases caused by soil-borne fungal pathogens in crop plants. It plays 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[1]. Analysis of the soil naturally suppressive to take-all of wheat, black root rot of tobacco, and tomato wilt revealed the prevalance of *Pseudomonas* spp. in their rhizosphere. *Pseudomonas* spp. is known to harbour *phl*ABCD locus responsible for the biosynthesis of phloroglucinol having antagonistic properties to pathogenic bacteria, which is highly conserved among different species of fluorescent Pseudomonads[2,3].

Phloroglucinol i.e. 2,4-DAPG is a phenolic polyketide with widespread antiviral, antibacterial, antifungal, anti-helminthic, and phytotoxic properties[4]. It possesses a strong antagonisitic activity against rhizosphere disease-causing pathogens in crop plants which makes *Pseudomonas* spp. a potent biological control agent[5,6]. The antagonistic activity of pseudomonas was confirmed by complementing mutants of pseudomonas that are deficient in pholoroglucinol production with the DAPG gene cluster. These transformed bacteria showed biocontrol activity upon transfer of DAPG biosynthetic gene cluster, confirming the role of DAPG in plant protection[1,7].

Genes necessary for biosynthesis of 2,4-DAPG spans over a genomic fragment of ~6500 bp in *Pseudomonas* spp and is organized into an operon harbouring six genes namely *phl*A, *phl*B, *phl*C, *phl*D, *phl*E, and *phl*F. Amongst all *phl*, *phl*ABCD are involved in the biosynthesis of DAPG, *phl*E is the efflux protein and *phl*F is the repressor protein. *phl*ABCD are indispensable and work in tandem for synthesis of phloroglucinol. *phl*D is involved in the biosynthesis of MAPG and *phl*ABC are essential for conversion of MAPG to DAPG[8,9]. Products of these genes resemble neither type I nor type II polyketide synthase (PKS) enzyme systems. Rather, *PhlD* shows similarity to plant chalcone synthases, indicating that phloroglucinol synthesis is mediated by a novel kind of PKS[10-12]. Most importantly the production of 2,4-DAPG by fluorescent *Pseudomonas* spp can be modulated by external stimuli such as glucose in many strains[13] or by sucrose or ethanol in a few strains[12-14].

Amongst all four *phl* genes viz; *phl*A, *phl*B, *phl*C and *phl*D; only *phl*C possess structural features

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typical of condensing enzymes. It also shows remarkable similarity with SCPx thiolase domain of plants indicating its role in plant defense. These features point towards the important role of *phl*C in DAPG gene cluster and also the overall role of DAPG gene cluster in plant defence. Here, we have made an attempt to clone, chracterize and do molecular analysis of *phl*C gene from a native Indian strain of *Pseudomonas* spp.

Materials and Methods

Genomic DNA isolation and PCR amplification of *phl*C gene

Genomic DNA was isolated from Indian strain of *Pseudomonas fluorescens* as described earlier[15]. The *phl*C gene from *Pseudomonas* spp was amplified and cloned by polymerase chain reaction strategy[16] using primer sets PhlCFP: AGCTCTAGAACCATCCAGCAAGGAGCA and *phl*CRP: GTGGGATCCTTAATCGTTG GAAAGCAC from the nucleotide sequence of *Pseudomonas fluorescens* (U41818) available at EMBL database. Restriction sites for enzyme *Xba*I and *Bam*HI were incorporated at 5' end of forward and reverse primer (underlined sequence), respectively. Restriction sites for *Xba*I and *Bam*HI were used as these restriction sites were not present internally in the *phl*C gene sequence. The PCR 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 100µl volume. The thermal cycling was performed with an initial denaturation cycle of 5 min at 95°C, followed by 30 cycles of (i) denaturation at 95°C for 1 min; (ii) annealing for 30 s at 60.5°C and (iii) extension for 1 min at 72°C and one cycle of final extension for 7min at 72°C.

Cloning of *phl*C gene in pBluescript (SK+) vector

To clone PCR amplified *phl*C gene into pBluescript (SK+) vector, PCR product (insert) and control pBluescript (SK+) vector DNA were double digested with *Bam*HI and *Xba*I simultaneously. The reaction mixture was incubated at 37°C for 3 h. Purified PCR product and linearized control vector were assessed on 1.2% agarose gel. Purified 50 ng linearized pBluescript vector, 100 ng double digested PCR product were ligated using T_4 DNA ligase. Mixture was incubated at 4°C overnight for ligation 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 (100 µg/mL) plate.

Confirmation of cloning and sequencing

Positive clones were confirmed by colony PCR with gene specific primers and restriction digestion with *Bam*HI/*Xba*I enzymes. The complete nucleotide sequence was determined by Sanger di-deoxy sequencing using universal forward (M13F) and reverse (M13R) primers as well as *phl*C gene specific primers. The final sequence was determined from both strands and comparison of *phlC* nucleotide and amino acid sequences were carried out.

Structure prediction

Model of PHLC was developed using I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/)[17] as per earlier report[18]. I-TASSER (Iterative Threading ASSEmbly Refinement) is a hierarchical approach to protein structure and function prediction. Structural templates were first identified from PDB by multiple threading approach LOMETS; full-length atomic models are then constructed by iterative template fragment assembly simulations. The generated model was refined using ModRefiner (http://zhanglab.ccmb.med.umich.edu/ModRefiner/). ModRefiner is an algorithm for high-resolution protein structure refinement. Both side-chain and backbone atoms were completely flexible during structure refinement simulations as per the algorithm of the software. ModRefiner allowed the assignment of a second structure that was used as a reference to which the refinement simulations were driven. The ModRefiner was used to draw the initial starting model of PHLC closer to its native state.

Ramachandran plot analysis

The stereo-chemical properties of PHLC were assessed by Ramachandran plot analysis using RAMPAGE[19]. It allowed visualization of energetically allowed regions for backbone dihedral angles ψ against φ of amino acid residues in PHLC protein structure. The residues in disallowed region were further refined using Modloop (https://modbase.compbio.ucsf.edu/modloop/)[20]. Modloop relies on MODELLER that predicted the loop conformations of PHLC by satisfaction of spatial restraints, without relying on a database of known protein structures.

Validation and visualisation of modelled structure

The validation of the modelled structure was performed using PDBsum[21] and PROCHECK[22].

Structure visualization was performed using Pymol. The predicted model of protein was submitted to Protein Model DataBase[23] (http://srv00.recas.ba.infn.it/PMDB/main.php).

Results

PCR amplification of *phl*C gene from genomic DNA of an Indian isolate of *Pseudomonas* as template resulted in a fragment of 1.2 kb (Fig. 1). The amplified PCR product and pBluescript control vector were then restricted simultaneously with *Bam*HI and *Xba*I restriction enzymes. This resulted in a 1.2 kb fragment PCR product with sticky ends (insert) and 3 kb linearized control vector pBluescript (SK+) with sticky ends for *Bam*HI and *Xba*I (Fig. 2).

The purified double digested PCR product (insert) was ligated with linearized pBluescript vector. The ligated mixture was transformed into *E. coli* DH-5α competent cells and five random white colonies were picked and used for colony PCR (Fig. 3A, Lane 2). Presence of phlC gene was further confirmed by restriction digestion with *Bam*HI/*Xba*I enzymes that released the expected fragment of ~1.2 kb (Fig. 3B, Lane 2).

Sanger sequencing of the cloned *phl*C gene revealed that it consisted of 1220 nucleotides with an open reading frame of 1197 bp. Based on the nblast (nucleotide BLAST) results, it was confirmed that the *phl*C gene was full-length with an open reading frame of 1197 bp coding for a 398 amino acid long peptide. The cloned *phl*C gene showed considerable homology with the other known *phl*C gene from *Pseudomonas flouresence* strain Q2-87 indicating a common descent. The deduced amino acid sequence of 398 amino acids (~43.5 KDa) showed significant similarity with the homologs of PHLC (Fig. 4).

For detailed structural analysis of PHLC protein, 3-D model was generated in I-TASSER server using the deduced 398 amino acids sequence (Fig. 5). The homology modelled initial structure was based on template crystal structure of *Trypanosoma brucei* SCP2-thiolase like protein (TbSLP) form-II (PDB entry 5ab5)[24]. PHLC model had a C-score of -0.64 and a TM score of 0.8±0.09.

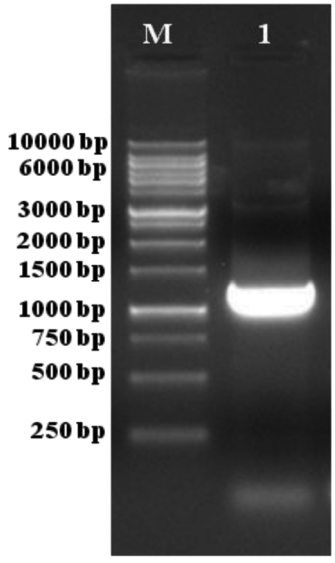

Fig. 1 — PCR amplification of *phlC* gene from genomic DNA of an Indian isolate of *Pseudomonas.* [Lane M: 1 kb DNA ladder (Fermentas); Lane 1: ~1.2 kb amplicon of *phlC* gene. Nucleotide sequence of *Pseudomonas fluorescens* (U41818) available at EMBL database was used for primer designing]

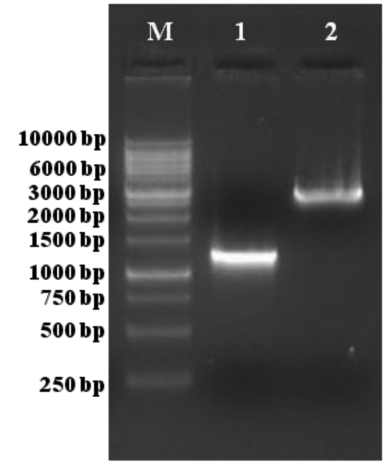

Fig. 2 — Restriction profile of double digested PCR product and control pBluescript vector. [Lane M: 1 kb DNA ladder (Fermentas). Lane 1: ~1.2 kb fragment of Purified PCR product digested with *Bam*HI/*Xba*I. Lane 2: ~3 kb fragment of pBS digested with *Bam*HI/*Xba*I]

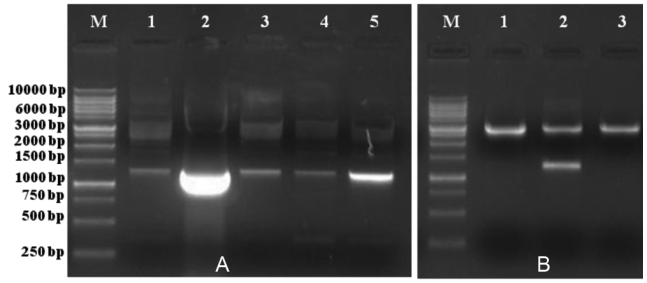

Fig. 3 — Screening and confirmation of positive putative clones by colony PCR and restriction digestion, respectively. (A) Colony PCR of positive colonies with gene specific primers. [Lane M: 1 kb DNA ladder (Fermentas); Lane 1-5: PCR amplified product. Only colony 2 yielded an amplicon of ~1.2 kb]; and (B) Restriction digestion of plasmid DNA isolated from putative *phlC* clones. [Lane M: 1 kb DNA ladder (Fermentas); Lane 2: Restriction digestion with *Bam*HI/*Xba*I released a fragment of ~1.2 kb of *phlC* gene and ~3 kb fragment of pBluescript (SK+) vector backbone]

This initial model was refined using modrefiner and Ramachandran plot analysis that revealed 90.4% residues in favoured region, but 2% residues were in outlier region. The initial model was iteratively refined using modloop until 0% residues fell in outlier region with 95.9% residues in favoured region and 4.1% in the allowed region (Fig. 6). The predicted model was submitted to Protein Model

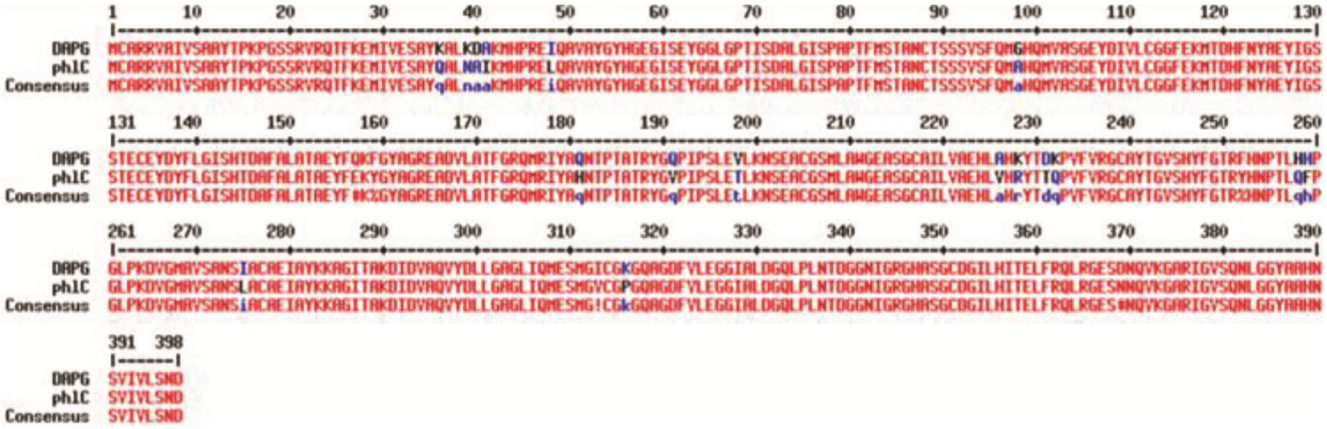

Fig. 4 — Pairwise sequence alignment of deduced amino acid sequence of PHLC protein with reported DAPG producing PHLC protein from *Pseudomonas* data base (http://multalin.toulouse.inra.fr/multalin/).

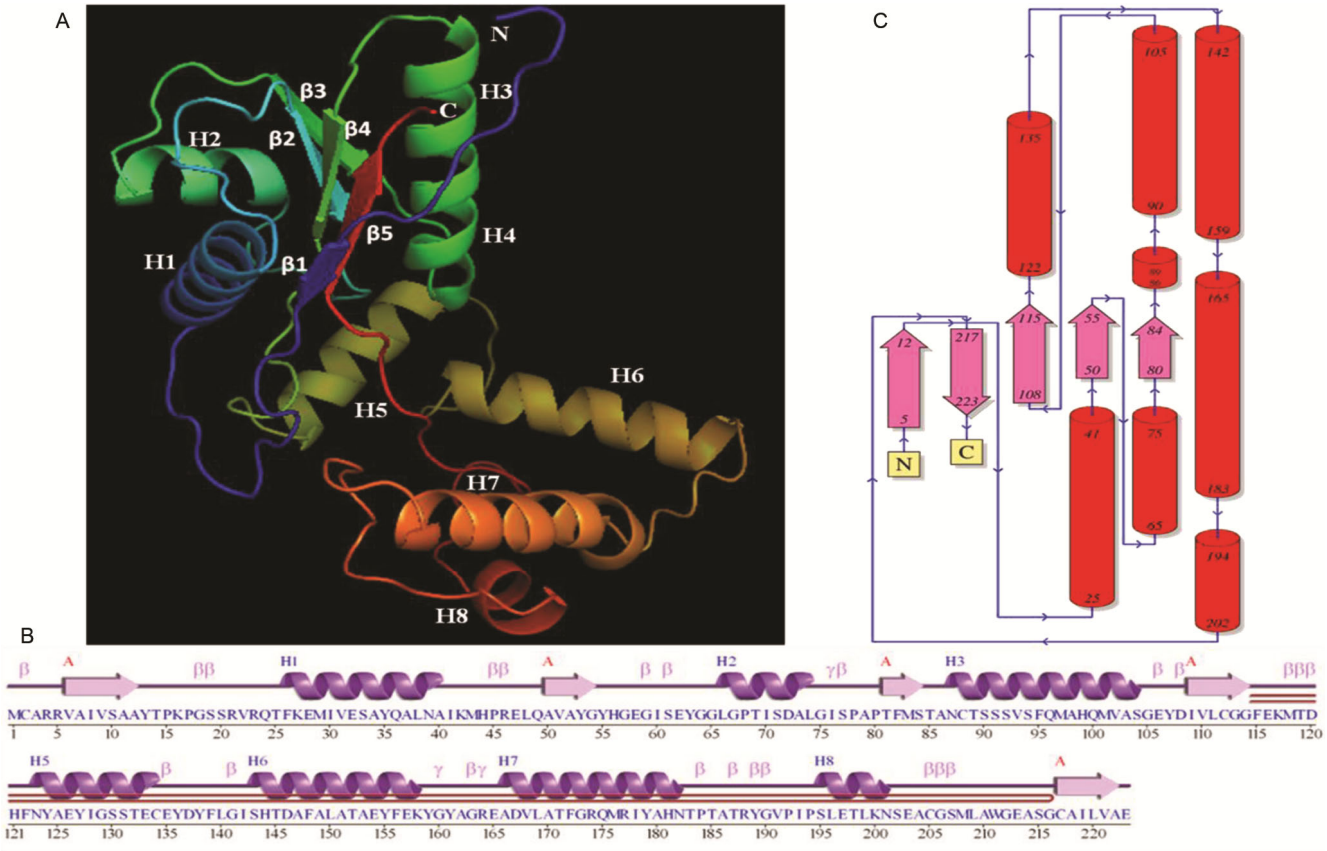

Fig. 5 — Structure of PHLC from *Pseudomonas spp*. predicted using I-TASSER server. (*A*) Stereo-ribbon diagram of the PHLC monomer (chain *A*) colour-coded from the N-terminus (blue) to the C-terminus (red). Helices (H1–H8) and β-strands (β1–β5) are indicated; (*B*) Diagram showing the secondary-structure elements of PHLC superimposed on its primary sequence. The labeling of secondary-structure elements is in accordance with *PDBsum* (http://www.ebi.ac.uk/pdbsum): α-helices are labeled H1 - H8, the β-strands are labeled β1–β5, β-turns and γ-turns are designated by their respective Greek letters (β, γ) and red loops indicate β-hairpins; and (C) Topology of PHLC protein showing the orientation of α-helices and β-strands.

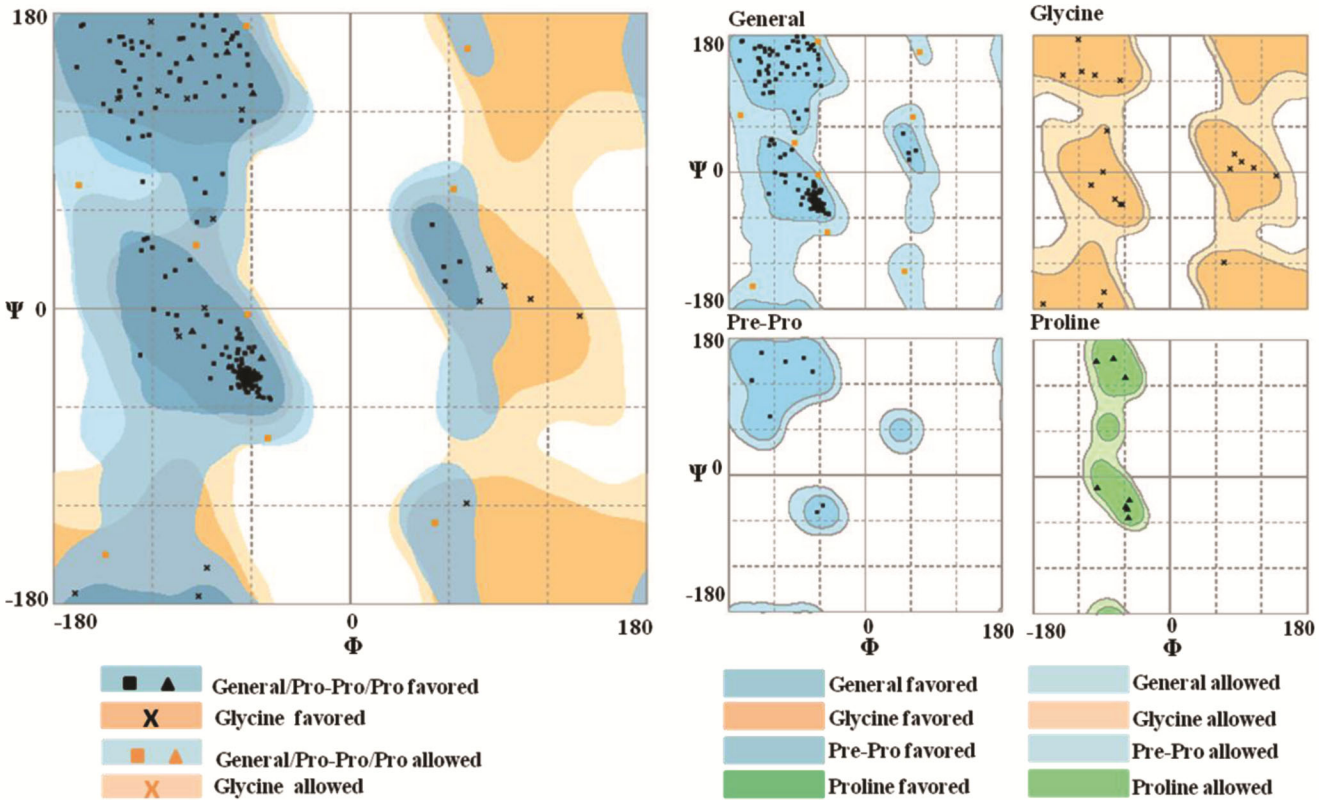

Fig. 6 — Stereochemical structure stability was analysed using Ramachandran plot analysis using RAMPAGE. [About 95.9% residues fall in favoured region and 4.1% in the allowed region]

DataBase (PMDB) and was assigned identifier PM0080924.

The three-dimensional model predicted by homology modelling was in accordance with the secondary structure predicted by PDBsum. PHLC structure revealed the presence of 1 beta sheet and 8 alpha-helices. β-sheet A contained 5 β-strands with topology 3X 1X -2X -1[25]. The protein also contained 8 α-helices viz; α1 {Phe26-Ala40 (15 residues)}, α2 {Leu66-Leu74 (9 residues)}, α3 {Asn87-Thr89 3 residues)}, α4 {Ser90-Ser104 (15 residues)}, α5 {Asn123-Cys134 (12 residues)}, α6 {Ser143-Lys158 (16 residues)}, α7 {Ala166-Asn182 (17 residues)} and α8 {Ser195-Asn201 (7 residues)}. One β-hairpin of class 105:105 was also identified[26]. Three helix-helix interactions between α1 & α2 (H-H type), α5 & α6 (H-H type) and α7 & α8 (H-H type) were identified. One β-α-β motifs with 26 loops and 11 helices participation was identified along with one β-bulges antiparallel wide type structure. Altogether, there were 23 β-turns belonging to three classes: Class I {(Gly18-Arg21), (His44-Glu47), (Ile76-Ala79), (Thr183-Ala186), (Ala186-Tyr189) and (Arg188-Val191)}, Class IV{(Met1-Arg4), (Pro17-Ser20), (Pro45-Leu48), (Glu58-Ser61), (Ile60-Tyr63), (Lys117-Asp120), (Met118-His121), (Thr119-Phe122), (Cys134-Asp137), (Leu140-Ser143), (Ala162-Glu165), (Tyr189-Pro192), (Ala204-Ser207), (Cys205-Met208) and (Gly206-Leu209)} and Class VIII {(Gly105-Asp108) and (Tyr107-Val110)}[27] were identified. Three γ turns at Gly75-Ser77, Tyr159-Tyr161 and Gly163-Glu165 (inverse type) were also identified.

Discussion

Green revolution marked an increase in the yield[28,29] of only food grains in past years, but other crops still need attention. Apart from abiotic stresses, there are many biotic stresses[30] which affect the yield of the plant. With the advances made in the field of genomic[31-33] and transgenics[34,35], alternative approaches to impart biotic/abiotic tolerance[36] or engineer a trait in crops[37] have been developed. Pseudomonads are a group of gram-negative bacteria known to produce antibiotic and antifungal compound known as "Phloroglucinol." It acts as a natural biological control agent against many plant pathogens causing diseases like damping off in sugar beet[14], black root-rot in

tobacco[2], etc. Biosynthetic operon responsible for synthesis of phloroglucinol compound i.e. 2,4–diacyl phloroglucinol in *Pseudomonas* spp is organised as a 6.5 kb genomic region in its genome. Till date complete genome sequence of many *Pseudomonas* species have been studied with an objective to delineate the structural details and understand the mechanism of regulation of 2,4–DAPG biosynthesis. Apart from promoting growth in-vitro[38,39], *Pseudomonas* spp. are reported to be involved in pigment production[40] and absorption of heavy metals[41,42]. Our investigation focussed on the cloning of *phl*C gene from a native Indian strain of *Pseudomonas spp* and identification of the *phl* locus responsible for DAPG production. We have successfully cloned the 1197 bp *phl*C gene from a native Indian strain of *Pseudomonas* spp. The translated nucleotide sequence comprises of 398 amino acids including the start codon. This is consistent with the earlier reports of *phl*C gene from different *Pseudomonas* strains like *Pseudomonas* sp. Q12-87, *Pseudomonas* sp. K96.27, *Pseudomonas* sp. PITR2, *Pseudomonas* sp. Q37-87, *Pseudomonas* sp. 12 and shares about 90-95% amino acid sequence identity[43].

Proteins coded by *phlC* gene along with *phlA* and *phlB* are necessary for the synthesis of MAPG and its conversion to 2,4-DAPG. Out of all three genes, only *phl*C contains structural features typical to a biosynthetic condensing enzyme involved in MAPG acetylation. The first structural feature is cysteine-88 of PHLC that aligns with the catalytic cysteine in the active site of thiolases (condensing enzymes) and may act as the binding site for acetyl-CoA prior to synthesis of acetoacetyl-CoA (putative monomer of malonyl CoA). A second conserved region included the sequence ^{346}GHASGCDG, (histidine underlined) found to be conserved in condensing enzymes and thiolases[44]. It is speculated that histidine forms a part of the binding site for a malonyl thioester, the extender unit (building blocks) in biosynthetic condensation reactions. However, it has been predicted that *phl*C generates acetoacetyl-CoA, as type II thiolases and differs from the latter in lacking a cysteine 378, a key active site residue[45]. Therefore, acetyl-CoA is the least favoured extender unit used by *phl*C. It, however, closely resembles the thiolase domain of SCPx, the product of an apparent fusion of genes for thiolase and a small mammalian protein important in the intracellular transport of lipids and sterols, particularly cholesterol[44,45]. Similarity between the SCPx thiolase domain and *phl*C extends not only in the key catalytic regions rather over the entire length of the sequence (Fig. 5), suggesting that the two proteins may have common conformational features, facilitating an interaction between *phl*C and MAPG similar to that between SCPx and its sterol ligands[43].

The three-dimensional structure of PHLC protein predicted by I-TASSER server was based on template crystal structure of *Trypanosoma brucei* SCP2-thiolase like protein (TbSLP) form-II (PDB entry 5ab5)[24]. PHLC shared all the features of thiolase with *Trypanosoma brucei* SCP2 such as weak binding or preference to acetyl CoA[24]. Also, PHLC showed similarity to SCPx thiolase and C-terminal part of SCPx was identical to SCP-2 (https://www.ebi.ac.uk/interpro/entry/InterPro/IPR002155/). This correlated PHLC protein with *Trypanosoma brucei* SCP2-thiolase like protein (TbSLP) (Fig. 5). PHLC model had an excellent C-score of -0.64 indicating a good quality model. C-score is a confidence score for estimating the quality of predicted models by I-TASSER. Its value usually ranges between −5 to 2 and a higher value indicates high quality of the model. Similarly, TM-score is a metric for assessing the topological similarity of protein structures. TM-score >0.5 indicated a model of correct topology and a TM-score <0.17 represents a random similarity. A TM score of 0.8±0.09 for predicted PHLC protein was indicative of correct topology. Also, occurence of >90% residues (95.9% residues) in favoured region of Ramachandran plot signified the stereochemical stability of the generated and refined molecule is of higher order.

Conclusion

In this study, we identified and successfully cloned the *phl*C gene from a native Indian strain of *Pseudomonas* spp. to 1197 bp in length. Additionally, we predicted the three-dimensional structure of PHLC protein and based on our results conclude that PHLC protein has all the features of a thiolase and is indispensable for the functioning of *phl* operon in *Pseudomonas*. Our findings may aid in discerning the role of *phl* locus in *Pseudomonas* as a potent biological control agent against rhizosphere pathogens in important agricultural crops. Further, reverse genetics approach of *phl*C (knockdown/gene silencing) would provide a detail insight about its crucial involvement in DAPG production in bacteria.

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Conflicts of interest

Authors declare no competing interests.

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